MICROBIAL DEGRADATION OF CYCLODIENE PESTICIDES

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Decomposition of the pesticides aldrin and dieldrin by aquatic microorganisms may result in more harmful products affecting the ecosystem. Isolates from fresh, brackish, and littoral water were cultured using, variously, selective media, enriched laboratory ecosystems, and impregnated wood strips and assessed qualitatively and quantitatively for action on pesticides by thin-layer chromatography, gas-liquid chromatography, and ^{14}C-labeled pesticide autoradiography. Growth was measured in terms of dry wt and cell nitrogen. Small amounts of possible metabolites were indicated in most isolate preparations. Cellulolytic Z. xylestrix grew well in media containing 10-500 ug/ml pesticide. Growth was stimulated by small quantities of the chemical and the fungus accumulated pesticides to levels 2000 times higher than the original medium concentration. Uptake increased linearly with increasing pesticide concentration. A significant portion of the pesticides appeared to be chemically altered, but the products were not specifically identified.

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Microbial Degradation of Cyclodiene Pesticides

by

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1 January 1974

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FORWARD

This constitutes the first full-length report on the progress made toward obtaining a microbial preparation capable of destroying aldrin and dieldrin waste residues in a manner compatible with operating force policies on pollution reduction. Tasked with this responsibility by the Program Director for Naval Biology approximately 1.5 years ago, it was necessary to phase out one line of investigation while phasing in the new assignment. The period of adjustment was necessarily lengthy.

Since our laboratory was primarily set up for metabolic and physiologic investigations of marine fungi, it was normal to include certain species of these in our screening for pesticide degradative activity. In addition, however, provision for other microorganisms, especially bacteria, was also made in the screening protocol. The emphasis continued to be on aquatic types. Since there was little in the literature concerning our particular interest, isolation and screening work began at the lowest level. A whole series of techniques new to this laboratory were incorporated from the literature or devised. Much of the following is concerned with this period of expertise development and initial results with various microflora. It is anticipated that work now in progress will add greatly to the substance of our initial experiences.

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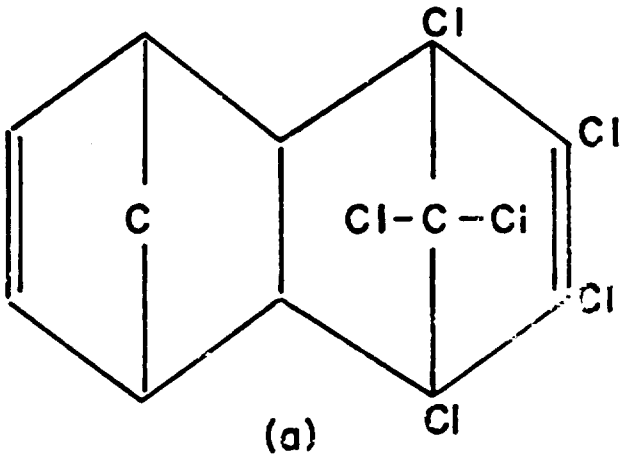
HISTORICAL INTRODUCTION

Increasing concern over the accumulation of organochlorine pesticides in soil and water, and the potential harmful effects thereof, has resulted recently in banning most uses of DDT throughout the United States (Gillette, 1972). Other closely related pesticides, although not banned completely, have been restricted extensively. One of the arguments used in the document banning DDT was the probable carcinogenic effect of the toxican. There is no convincing evidence to date that either DDT, or its related organochlorine pesticides, cause cancer. In fact, there is no factual contention that current levels of pesticides in surface water or foods present an acute toxicity hazard to man (Zavon, 1970). Yet, increased urinary excretion of D-glucaric acid - a well-recognized hepatic microsomal enzyme activity indicator - has been reported in men engaged in the manufacture of endrin (Hunter et al., 1972). This may point to a neoplastic effect of long term exposure.

According to Matsumura (1972), an estimated 10 billion pounds of pesticides have been used in the United States since 1945. Whether man eventually will be physicially harmed by the accumulation and recycling of pesticides remains the subject of much controversy. Much less disagreement exists with respect to the effect on nontarget organisms important in food chain sequences. The detrimental effects of long-term, low-level environmental exposure, particularly in primary producers, such as

phytoplankton (Ware and Roan, 1970), continue to place concern not on the future use of pesticides, but on the interaction that may result with environmentally persistent chemicals already applied. Reports, such as those on the massive Mississippi fish kill (Biglane, 1964), Clear Lake (Rudd, 1964), and the Richdale-Colusa study (Cottam, 1965), have gained national attention and brought into the public arena the controversial cause celebre of Rachel Carson's Silent Spring (1962).

Among the chlorinated hydrocarbon pesticides, the cyclodienes aldrin and dieldrin (Fig. 1) have found extensive use in replacing DDT, particularly where target organisms have become resistant. O'Brien (1967) has indicated that these chemicals act as neurotoxicans with a mode of action similar to, but slower than DDT. Perhaps the most distinguishing general characteristic of organochlorine pesticides is their persistence in the environment. Edwards (1966) gives a 95% persistence rating for aldrin of 1-6 yr, with an average of 3 yr, while dieldrin persists for 5-25 yr with an average of 8 yr. Monitoring data in the U.S. show that DDT, its conversion products DDD and DDE, and dieldrin are the most highly concentrated and commonly found organochlorine pesticides (Edwards, 1970). Most studies have shown also that bottom sediments of water systems act as the largest reservoir of organochlorine pesticides (Lichtenstein et al., 1966). On the other hand, use of ultra-low volume spraying methods have contributed greatly to the broad dispersion of these chemicals by wind. Dust,



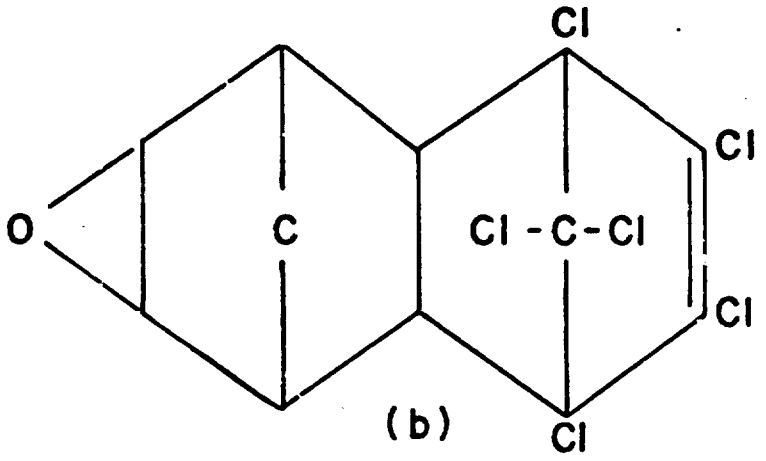


FIG. 1. Planar configurations of (a) aldrin and (b) dieldrin.

collected by Riseborough (1968) over Barbados, contained approximately 41 ppb of pesticide which could have indicated a deposit of 600 lb/yr on that area of the Atlantic Ocean between the equator and 30° North, as a result of rainfall.

Bioconcentration, or pesticide residue accumulated by an organism by adsorption and by absorption via oral or other route of entry (Kenaga, 1972), seems to play a major role in the environmental persistence of these pesticides. Other factors influencing persistence are volatilization, photodecomposition, chemical decomposition, co-distillation, and microbial attack. None, however, has stirred so much controversy as microbial decomposition. The "infallibility principle" of Gale (1952), first questioned by Alexander (1965), has gained new attention in a recent review by Horvath (1972). He concluded that failure to demonstrate biodegradation of so-called "recalcitrant" molecules is perhaps more a consequence of human technological inadequacy than of microbial inability. In spite of this, considerable information has been obtained regarding interaction of organochlorine pesticides with soil microorganisms and, to a lesser degree, aquatic types.

As early as 1954, Fletcher and Bollen examined three Oregon soils and six soil classes, with aldrin applied at a concentration of 1000 and 2000 ug/ml. Aldrin appeared to stimulate bacterial populations with the exception of streptomyces. Fungi appeared unaffected. The toxicity of DDT, chlordane, HHC, dieldrin, aldrin, and endrin for soil

microorganisms was examined by Jones (1956) who found that none of these compounds was excessively inhibitory to ammonifying forms. Dieldrin and aldrin, however, were among the most toxic compounds and inhibited nitrifying microorganisms at concentrations of 0.1%. Eno and Everret (1958), utilizing ten two-gallon glazed pots filled with sand, at pH 6.65, and ten pesticides, including aldrin and dieldrin, concluded that application of as much as 200 lb/acre of most pesticides caused little or no damage to the ten microbial populations. They relied upon measurements of CO_2 evolution and total numbers of fungi and bacteria. The data even suggested that microorganisms may be stimulated by the addition of such compounds. In another study, Martin (1969) concluded that aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, lindane, and toxaphene exerted no measurable effect on the number of soil bacteria and fungi, on kinds of soil fungi developing, or on the ability of the soil population to perform the normal functions of organic matter decomposition and ammonia oxidation. The reports of Collins (1968) and Langlois (1970) concerning the effects of DDT, dieldrin and heptachlor on the growth of selected bacteria showed that dieldrin had no effect on the growth of the organisms. Lichtenstein and coworkers (1966) noticed that aldrin was broken down rapidly in active lake and pond sediments, but not in autoclaved or sodium azide-treated mud-water systems.

The major concern in aquatic environments has centered on the interaction of pesticides with phytoplankton.

Cairns (1968) reported that the diatom Navicula seminulum was able to survive at a concentration of dieldrin considerably greater than that reported as harming fish and aquatic invertebrates. Wurster (1968), however, concluded that as little as a few ppb of DDT in water reduced photosynthesis in laboratory cultures of four species of coastal and oceanic phytoplankton representing four major classes of algae. Also, toxicity to diatoms increased as cell concentration decreased. Photosynthesis and growth of four marine phytoplankton species, isolated from different oceanic environments, were investigated by Menzel, Anderson, and Randtke (1970) and reported to be affected variously by 0.1-10 ppb of DDT, dieldrin, and endrin. Dierksheide and Pfister (Dierksheide, personal communication) studied the morphological and physiological responses of two blue-green bacteria to aldrin and dieldrin. Growth of Anacystis nidulans was inhibited initially by aldrin and dieldrin. On continued incubation, however, the organism recovered. Oxygen production was inhibited only by dieldrin after a delay of 24 hr. Aldrin and dieldrin appeared to act through different mechanisms of inhibition in A. nidulans. Aldrin inhibited the amount of chlorophyll synthesized; dieldrin suppressed photosynthetic activity in an unknown manner. Cell numbers were reduced by single or daily additions of dieldrin to cultures of Microcystis aeruginosa. Pesticides were concentrated by both organisms but, at the levels employed, it was impossible to identify the sites of inhibition with the electron microscope.

Batterton, Boush, and Matsumura (1971) found that metabolic products of aldrin, dieldrin, and endrin could be as inhibitory to algal growth as the parent compounds.

Soil and sludge microorganisms on the other hand, have been sought more actively for biodegradation activity. Chacko and Lockwood (1967) demonstrated the accumulation of dieldrin by a number of fungi and bacteria. Hill and McCarthy (1967) found that aldrin, among other organochlorine pesticides, was degraded rapidly in sewage sludge under anaerobic conditions. Dieldrin was also degraded, but much more slowly. Tu, Miles, and Harris (1968) studied the action of soil microorganisms on aldrin and heptachlor and reported that, in most cases, conversion of aldrin to dieldrin, and heptachlor to heptachlor epoxide occurred. Wedemeyer (1968) showed that the ubiquitous bacterium Enterobacter aerogenes (Aerobacter aerogenes) converts dieldrin to 6,7-trans-dehydroxydihydro aldrin. Matsumura and co-workers (1967, 1968, 1971) isolated from pesticide-enriched soil a number of microorganisms that degraded dieldrin and endrin. This group, at the University of Wisconsin, reported that the fungus Trichoderma koningi is capable of degrading one or more of the carbons on the chlorinated ring of the dieldrin molecule to CO_2 (Bixby et al., 1971).

Some of the organisms used in studies involving chlorinated hydrocarbon pesticides were isolated from industrial effluents (Bourguin et al., 1972), ponds, lakes, and natural streams. However, interaction with autochthonous

marine microorganisms in particular, and the fate of these pesticides in aquatic environments in general, has received very little attention.

It was the purpose of this investigation to establish methods for the study of interactions between the cyclodiene organochlorine pesticides, aldrin and dieldrin, and microorganisms isolated from aquatic environments, as well as autochthonous marine microorganisms presently held in stock; and to assess the capabilities of these isolates for altering the concentration of these pesticides in liquid media either by accumulation or transformation.

MATERIALS AND METHODS

Reagents. All organic solvents used in the extraction of pesticides were Nanograde (Mallinckrodt Co.), Pesticidequality (Matheson, Coleman & Bell Co.), or redistilled in a glass apparatus and dried over anhydrous Na_2SO_4. Analytical reagent grade chemicals were employed for the preparation of synthetic media and other solutions. Analytical aldrin,1,2,3,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-5,8-dimethanonaphthalene, (HHDN) 99.5%, and dieldrin, 1,2,3,4,10,10-hexachloro-6,7,epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-exo-5,8-dimethanonaphthalene, (HEOD) 99.5%, were generously provided by Shell Chemical Co., Agricultural Chemicals Division. Highly purified, gas-liquid and thin-layer chromatography analyzed aldrin, dieldrin, and lindane (1,2,3,4,5,6-hexachlorocyclohexane) were obtained from the Pesticide Repository (Environmental Protection Agency), Perrine, Florida. Technical aldrin and dieldrin, as well as uniformly chlorinated ring-labeled ^{14}C-aldrin (99.6% pure, specific activity 2.42 mCi/mmole) and ^{14}C-dieldrin (99.6% pure, specific activity 2.6 mCi/mmole) were also supplied by Shell Chemical Co. Florisil, 60/100 mesh factory-activated at 600 C, was obtained from Fisher Scientific Co. and kept at 130 C. Deionized water (DW), obtained from a Barnstead Demineralizer (standard cartridge), was used throughout these studies. Artificial sea water (ASW) was prepared according to Lyman and Fleming (1940).

Unless otherwise stated, the pH of media and other solutions was adjusted with either 6 N HCl or 10 N KOH; autoclaving was done for 15 min at 15 psi; cell homogenates were obtained by blending in Waring stainless steel semi-micro containers; all analytical results represent averages of duplicate or triplicate determinations.

Field Stations. Suitable stations for the collection of water and sediment and for implanting isolation systems were chosen from the surrounding area and monitored for physical and chemical parameters. Temperature was recorded with a calibrated thermometer. Salinity was measured by comparing the specific gravity obtained with a hydrometer to a previously prepared standard curve. Oxygen content was estimated with a modified micro-Winkler method (Clark, 1960) and pH readings were obtained, in the laboratory, with a standardized Beckman Zeromat SS-3 pH-meter. Surface water samples, for analysis of residual aldrin and dieldrin, were collected in wide-mouth, 1900-ml, screw-cap glass bottles (8.5 by 21 cm) and stored at 4 C until extracted (1-4 months). Except for station SP, all samples were obtained from locations in Broward or Palm Beach Counties, Florida. Station SP consisted of two sediment cores (ca. 25 g), one surface water sample (ca. 50 ml), and a mixed sediment-surface water sample (ca. 50 ml) received from Dr. Harold P. Vind of the Naval Civil Engineering Laboratory, Port Hueneme, California. These samples were obtained 1-3 km from shore in 10.5 m of water, 1.6 km from the end of the main ocean outfall from the Whites Point Sewage Plant of

the County of Los Angeles. The amount of sample received in the mail, without refrigeration, was sufficient only for microbial isolation.

Isolation systems. Three isolation systems were employed.

System A: Surface water samples were collected according to the methods described in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (APHA, 1971) using sterile, wide-mouth, 500-ml, screw-cap glass bottles (6.5 by 13 cm). Sediments from the same areas were collected with a sampling device described by Barkley (1971) (Fig. 2). The cores, 5 by 13 cm (ca. 900 g) were extruded into, wide-mouth, 950-ml screw-cap glass bottles (7 by 16 cm). Four surface water and two sediment cores were obtained from each locale and rapidly brought to the laboratory. The samples were processed for isolation of microorganisms immediately.

System B: This system, suggested by C. M. Tu, Research Institute, Canada Department of Agriculture (personal communication), consisted of a 40-liter Pyrex carboy (Fig. 3) filled with approximately 10 kg of sediment and 10 liters of surface water. The amount of sediment and surface water was approximated from the number of cores or water samples added. The carboy was brought to the laboratory and incubated at 25 C, in fluorescent light, on a rotary shaker (New Brunswick, Model G33) at 60 rpm. Technical grade aldrin and dieldrin in ethanolic solution, containing 50 mg/ml aldrin and 25 mg/ml dieldrin,

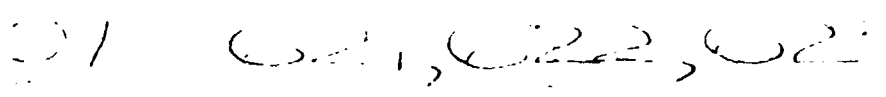


FIG. 2. Sediment sample collector assembled with coring terminus as employed for System A.

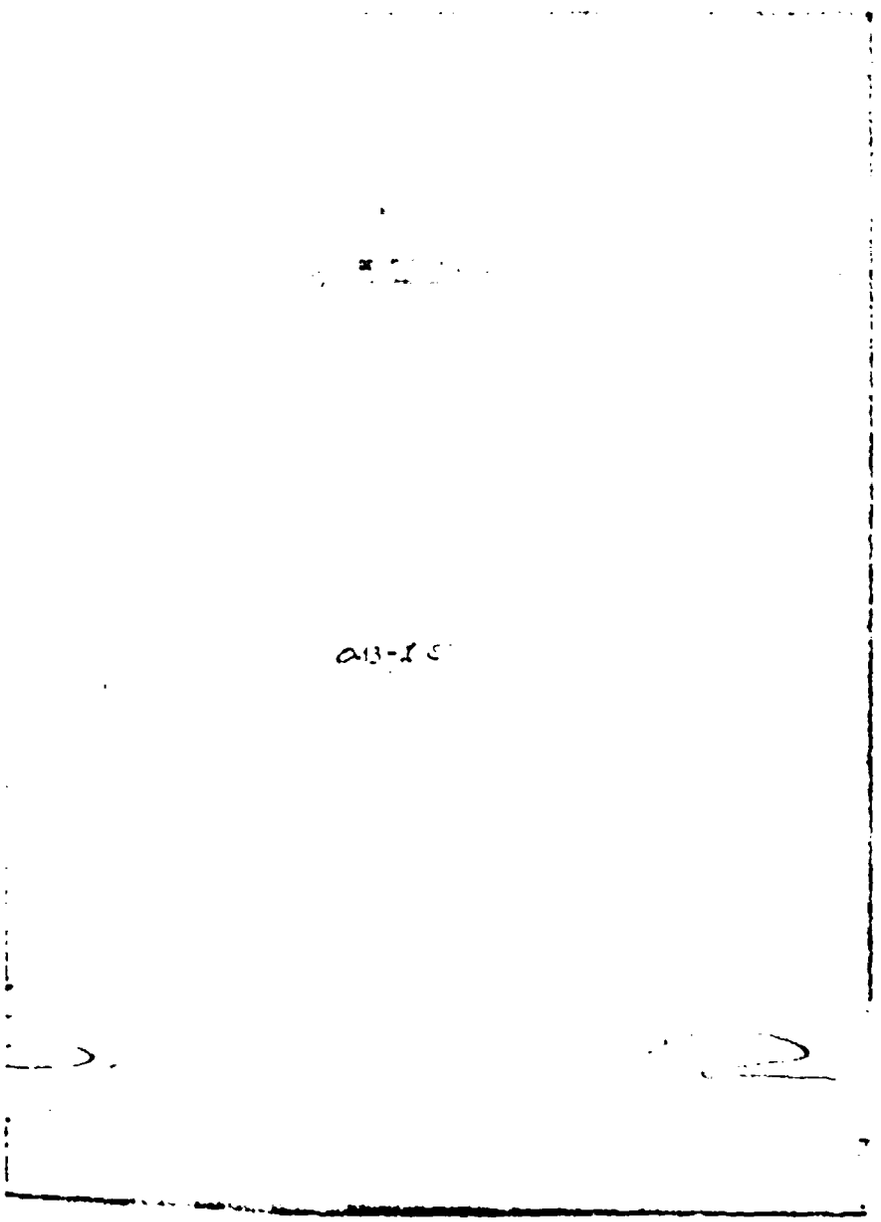


FIG. 3. Laboratory ecosystem of the type used in System B. The 40-liter carboy contained approximately 10 kg of sediment and 10 liters of surface water.

were added in 5 ppm increments, at 5 day intervals, until the concentration of each pesticide reached approximately 20 ppm (20 days). One month following the final addition of pesticides, the carboy was sampled using a 1 by 60 cm sterile glass tube. Several cores (ca. 10 ml) were removed at each sampling period and pooled into sterile, wide-mouth, 100-ml, screw-cap glass bottles (4 by 9 cm). The contents of the bottles, representing a uniform vertical core of the system (water and sediment), were processed for microbial isolation. One carboy was incubated for 4 months and sampled at 20, 41, 69, and 97 days. The samples obtained were inactivated with 0.5 ml of 20% trichloroacetic acid (TCA) and stored at 4 C for pesticide analysis. Macroscopic and microscopic observations of predominant microflora were made at each sampling time.

System C: A modification of the screened cages used by Vind (1971), this system consisted of canisters made from 6 by 23 cm polyvinylchloride (PVC) pipe with male and female adapters at each end (Fig. 4). The canisters were perforated with 36 holes, 0.3 cm in diameter, 2 cm apart. Each canister contained twelve 0.5 by 5 inch balsa wood strips impregnated with technical aldrin and dieldrin. The strips were immersed overnight in a 0.6% solution of both pesticides in 75% acetone. It was roughly estimated that the concentration of pesticides in the strips was 10 ppm. The canisters were submerged in chosen locations by means of cement or iron weights.

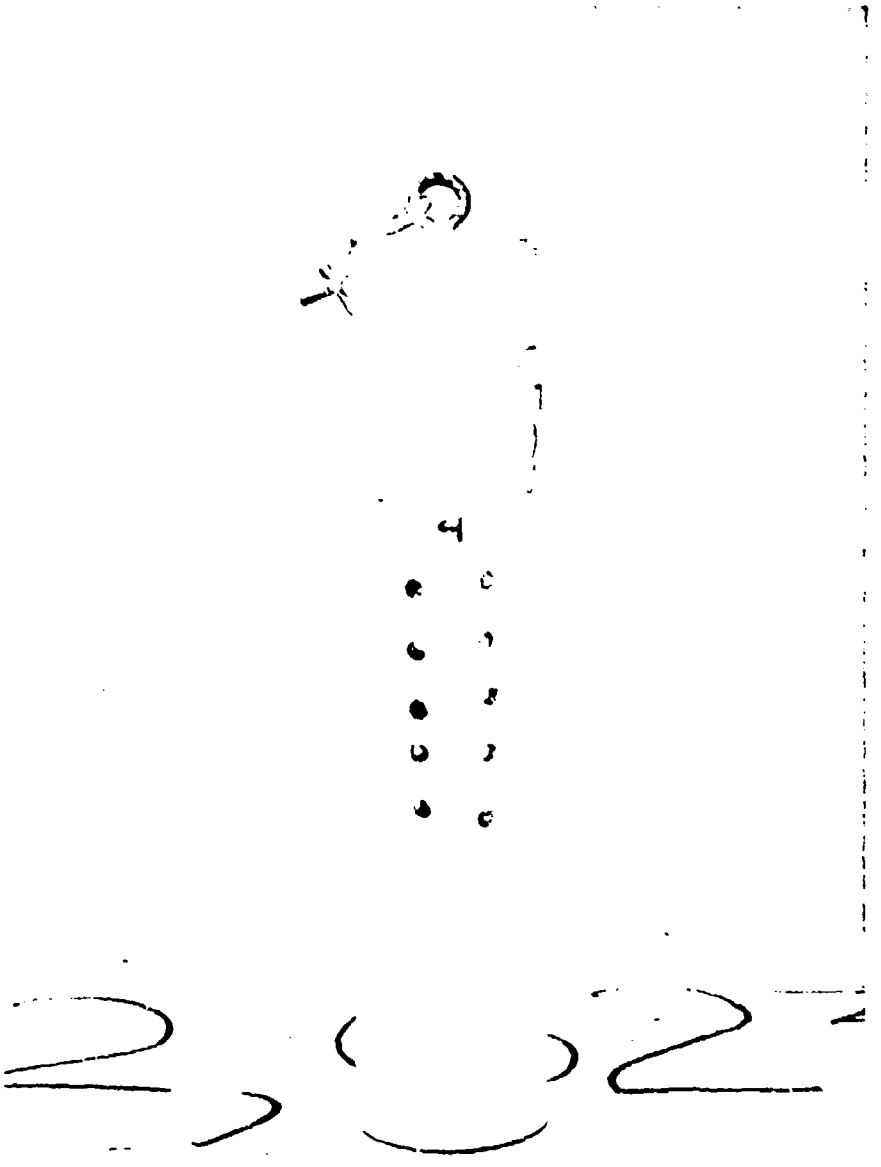


FIG. 4. PVC canister containing 12 balsa wood strips impregnanted with aldrin and dieldrin as used in System C.

Each canister and anchor was attached with No. 10 electrical wire to a styrofoam buoy which had an identifying copper tag secured to a 15 by 30 cm board. After one month the canisters were removed and transported to the laboratory in sterile, 15-liter glass jars. The canisters were opened aseptically and the strips from each placed in 2-liter Pyrex conical culture flasks containing 100 ml sterile DW. Next, the flasks were shaken for 15-20 min and immersed in a Branson Sonogen-Z ultrasonic cleaning bath for 10 min. The resulting supernatant liquid was used for microbial isolation.

Stock collection culture. The filamentous fungus used in this study was the Deuteromycete Zalerion xylestrix (FAU accession no. 2500) (Moore and Meyers, 1962).

Growth and isolation media. Organisms collected were isolated in the following media; except where otherwise mentioned pH was adjusted to 7.0 prior to autoclaving.

Selective AC (Isao and Thieleke, 1966): Peptone 0.5 g, glucose 1.5 g, KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, $Fe_2(SO_4)_3.9H_2O$ (trace), agar 17 g, DW or 75% ASW to 1 liter. After autoclaving, 0.5% of sterile mycostatin (Calbiochem) was added at 50 C. The medium was aseptically dispensed in sterile 10 by 150 mm plastic Petri dishes.

Selective F (Aaronson, 1970): Glucose 0.05 g, peptone 0.05 g, yeast extract 0.05 g, agar 30 g, DW or 75% ASW to 1 liter. After autoclaving, 1 g of streptomycin sulfate (Calbiochem) and 100,000 units penicillin G (Calbiochem) were added at 50 C. The medium was aseptical-

ly dispensed in sterile 10 by 150 mm plastic Petri dishes.

Enriched M4 (Booth, 1971): $(NH_4)_2SO_4$ 0.5 g, L-asparagine 0.5 g, KH_2PO_4 1.0 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.2 g, $CaCl_2$ 0.1 g, yeast extract 0.5 g, agar 15 g, DW or 75% ASW to 1 liter. After autoclaving, 100 ug/ml each of technical aldrin and dieldrin in acetone were added at 50 C. The medium was aseptically dispensed in sterile 10 by 100 mm plastic Petri dishes or tubed in appropriate volume without agar.

Minimal H (Shimakara and Yamashika, 1967): The basal medium contained NH_4NO_3 2.5 g, $MgSO_4.7H_2O$ 0.2 g and K_2HPO_4 1.0 g in 1 liter of DW or ASW containing 1.21 g tris(hydroxymethyl)aminomethane (THAM). For isolation and test purposes the complete medium contained 0.5% Tween 80. After autoclaving, the medium was aseptically dispensed in 10 ml amounts in 16 by 50 mm, screw-cap culture tubes. The same medium, containing 1.5% agar, was aseptically dispensed in sterile 10 by 100 mm plastic Petri dishes. Technical aldrin or dieldrin (100 ug/ml) in acetone were added to the liquid, or to the melted agar medium at 50 C, before dispensing.

Fungal 1410 M Basal (White, 1972): NH_4NO_3 2.5 g, THAM 1.21 g, yeast extract 1.0 g, Tween 80 5 g, ASW to 1 liter, pH 7.5.

Microbiological methods. Water samples from all stations and systems were passed through Millipore HA 0.45 filter membranes which were then suspended in 25 ml of sterile DW

and shaken vigorously for 10-15 min, or until the filters had practically disintegrated. Ten-fold dilutions to 1:1000 in sterile DW were made from these suspensions and 1.0 ml samples of the original suspension, and of each dilution, spread on each selective or enriched medium agar surface with a glass rod. One ml of original suspension was inoculated into 10 ml of minimal medium H, and 1 ml subcultured in fresh H media three times, at 5 day intervals. One ml of the last transfer served to inoculate a 10 by 100 mm agar plate of this medium. Approximately 10 g (wet) of sediment were suspended in 25 ml of sterile DW and treated as above without Millipore filtration. Plates were incubated at 25 C for 3-15 days.

Ten or more distinct colonies growing in any inoculated plate were transferred to fresh medium for confirmation. Cultures were then stocked under sterile mineral oil in either nutrient agar (NA) butts (for nonfilamentous forms) or Sabouraud dextrose agar (SDA) butts (for filamentous forms) and transferred every 6 months. Organisms growing on H media agar were transferred periodically (10-15 days) in H media broth and incubated statically at 30 C. These stock cultures were later transferred in F4 enriched media supplemented with 0.01% glucose (filamentous forms) or 0.01% peptone (nonfilamentous forms).

The choice of DW or 75% ASW was determined by the salinity at the place of isolation. Samples collected at stations with salinities of less than 1.5% were processed in DW media. Other media were prepared in 75% ASW.

Accordingly, organisms isolated from these stations were transferred and tested in corresponding media made either with DW or 75% ASW.

Cultivation and standardization of inocula for Z. xylestrix was achieved by a modification of the method described by Sguros, Meyers, and Simms (1962). Inocula were started from late linear (5 day) mycelia, homogenized for 35 sec in sterile semi-micro blenders, by inoculating 3 or 4, 125-ml flasks containing 100 ug/ml of either technical aldrin or dieldrin in 25 ml of 1410 M Basal medium. One ml of homogenate (ca. 2 mg dry mycelium) was used to inoculate each 25 ml culture. Cultures were incubated at 25 C on a New Brunswick, Model R-94 shaker, reciprocating at 66-8 cm strokes per min (spm). After 15-20 days, when heavy growth (ca. 2 mg/ml dry wt) was obtained, the media were pooled and homogenized for 5 sec. Twenty-five ml of homogenate served to inoculate 200 ml of 1410 M Basal medium containing either 100 or 500 ug/ml of aldrin or dieldrin in 1-liter conical flasks. Cultures started in aldrin were subsequently grown only in aldrin; dieldrin-grown cultures were restricted to dieldrin. Cultures restricted to glucose were treated similarly except that 100 ug/ml of glucose was added to the 1410 M Basal medium. The fungus was transferred every 15 days, after homogenizing the whole culture for 5 sec and inoculating 500-ml flasks containing 100 ml of fresh medium with 25 ml of homogenate.

Experimental inocula were started by inoculating 100 ml of 1410 M Basal with 25 ml of homogenate and starving

the culture for 10 days on the shaker at 25 C. Next, the whole culture was Millipore-filtered aseptically and the resulting mycelial pad washed three times with 50 ml volumes of sterile DW. The washed mycelial pad was then suspended in 50 ml of DW and homogenized for 5 sec. Twenty-five ml of the homogenate served to inoculate 500 ml of 1410 M Basal medium containing 100 ug/ml of glucose. After 10 days on the shaker, mycelia were Millipore-filtered and washed three times with 50 ml DW. The resulting pad was suspended in 50 ml DW and homogenized for 5 sec. Three 1 ml samples were filtered on tared Whatman GF/A discs for quantitation.

The discs were dried at 60 C in vacuo overnight and the remaining homogenate stored in the blender at 4 C. After the wt of the mycelia was established, the starved suspension was diluted with water to bring the mycelial concentration to approximately 2 mg/ml, and homogenized for 5 sec to ensure uniformity. Extra flasks were inoculated to provide a continuous source of viable inocula for future experiments. Cultures were checked routinely for contamination by means of microscopic wet mount preparations, Gram stains, and plating in suitable agar media.

Growth and characterization studies. Mixed group cultures were deliberately prepared from isolates, in the following manner: Organisms were mixed in groups of 8-12 according to isolation station and filamentous or non-filamentous form. Stock cultures, kept under oil, were transferred to fresh NA or SDA and incubated at room tempera-

ture for 1-3 weeks. A loopful of each culture was used to inoculate 25 ml of F4 medium, supplemented as previously described, and grown for 1 week on the reciprocating shaker at room temperature. One ml of the mixture was then used to inoculate 25 ml of H medium containing 100 ug/ml of both aldrin and dieldrin in 125-ml conical, screw-cap culture flasks. The cultures were shaken at 25 C and sub-cultured twice at 5 day intervals using 1 ml of inoculum each time. Five days after the last transfer, 0.5 ml of each culture was plated on H-agar medium and incubated for 1-3 weeks at 25 C. Growing colonies were transferred to fresh medium and incorporated into the stock group of isolates. All isolates resulting from plating on H medium, were transferred as described above, first on H media agar and later in liquid F4 media, supplemented with either glucose or peptone.

For all subsequent experiments, culture inocula were obtained as follows: A loopful of liquid medium culture was used to inoculate an agar slant of the same medium lacking pesticides. The slants were incubated 2-7 days at 30 C, 5 ml of sterile DW added to each, and the tubes swirled gently to dislodge the cells. This treatment produced a suspension containing approximately 10^8 cells/ml as estimated from the absorption of a 1:10 dilution at 490 nm in a Spectronic 20 colorimeter ($BaSO_4$ standard curve). Identical treatment of slants of filamentous organisms produced a suspension containing approximately 1 mg dry mycelium /ml. Cultures were checked routinely for contamination and

viability.

Partial characterization was achieved by: (a) Gram's stain (Hucker modification), (b) Leifson's flagella stain, (c) slide cultures (Talbot, 1971), (d) catalase and cytochrome oxidate tests (PathoTec Co., Warner-Lambert Co.), (e) growth on H medium "basal" containing 0.5% Tween 80, and either ethanol, acetone, glucose, aldrin, or dieldrin, (f) growth on Leifson's MOF medium (Difco) with 1% glucose, sucrose, or lactose, and (g) gelatin liquefaction (BBL). The examination was carried out, during incubation at 30 C, for 1-3 weeks.

Growth responses in Z. xylestrix were measured as the increase in dry mycelial wt or total cell nitrogen (N) in 1410 M Basal medium containing pesticides. Control cultures contained 10, 100, and 1000 ug/ml glucose without pesticides. Whole cultures were filtered through tared Whatman GF/A discs, washed liberally with DW and then with 50 ml of 50% acetone to remove any entrapped, undissolved pesticide. The discs were dried and weighed as before.

Mycelial N was determined by a micro-Kjeldahl method adapted from Johnson (1941). Samples, varying in volume from 5-15 ml obtained from 5 sec homogenates, were filtered and washed liberally with DW. The pads were resuspended in a minimum volume of water. One to five ml of cell suspension (depending on N content) was placed in 10-ml Kjeldahl flasks containing 1 ml of 9 M H_2SO_4 and digested over a burner until white fumes of SO_3 filled the vessels. The flasks were cooled for 0.5 min, 2 drops of 30% H_2O_2

added directly to the liquid and the mixture heated for an additional min to complete digestion. Next, the digested samples were transferred, by repeated 5 ml DW washings, to 50 ml Folin-Wu digestion tubes and diluted to 35 ml with DW. After adding 4 drops of 2% aqueous gum ghatti, the volume was made up to 50 ml with Nessler's reagent, and the ingredients quickly mixed. The tubes were read at 490 nm, within 10 min, in the Spectronic 20 and N calculated by converting the absorption readings to ug/ml ($(NH_4)_2SO_4$ standard curve). Controls invariably included the usual blank, standards, and cell suspension of known N value.

Extraction procedures. The analyses of water samples were carried out using methods and equipment available at the Primate Research Laboratories, Perrine, Florida (see Appendix I). The particular gas chromatography used at Perrine has been fully described by Thompson (1969). Analyses of water and sediment from isolation System B, station 04, were carried out by a modification of the method of Langlois, Stemp, and Liska (1964) because of high sediment moisture content (Hill and McCarty, 1966). Inactivated (TCA) samples were mixed and duplicate water and sediment composites placed in 50-ml conical centrifuge tubes. Each was then treated with 20 ug/ml analytical lindane to provide an internal standard. After centrifuging in a swing-bucket, size 2, International Centrifuge model K, at 1500 rpm for 30 min, the supernatant was decanted into a 250-ml separatory funnel. Extraction was done for 1 hr with 50 ml of hexane, on the reciprocating shaker

(66-8 cm spm) at 25 C. The aqueous layer was discarded, the hexane layer washed twice with 100 ml of DW, decanted into a 125-ml Erlenmeyer flask, and dried over anhydrous Na_2SO_4. A 25 ml sample was removed to a 35-ml graduated conical centrifuge tube and concentrated to 10 ml in a stream of N_2. No further purification was necessary for gas-liquid chromatography (GLC) using a flame-ionization detector (FID). The remaining solids were transferred to a large mortar and ground with 10 g of 5% deactivated Florisil. The mixture was poured on 10 cm deactivated Florisil covered by 2.0 cm anhydrous Na_2SO_4, in a 22 by 330 mm chromatographic column, previously washed with 50 ml of 20% dichloromethane in petroleum ether. The sample was eluted first with 100 ml of 20% dichloromethane in petroleum ether and then with 100 ml of 50% dichloromethane in petroleum ether. The eluate was collected in a 250-ml Erlenmeyer flask and dried over anhydrous Na_2SO_4. The whole extract was transferred to a 250-ml Kuderna-Danish evaporative concentrator assembled with a 10 ml receiver and a 3-ball Snyder column, and evaporated to approximately 3 ml in a boiling water bath. The assembly was removed, cooled, and the joints and spheres rinsed twice with 2 ml portions of hexane. Finally, the concentrated sample was evaporated to dryness in a stream of N_2, the residue dissolved in 5 ml of hexane, and held on a Vortex mixer for 1 min. Water and sediment extracts were pooled together and analyzed by GLC/FID (see p. 26), and the percent recovery estimated against the lindane internal standard.

After GLC analysis, a 3 ml sample was evaporated to dryness and the residue dissolved in 0.5 ml of hexane. The resulting solution was applied to the top of 3.0 cm of 5% deactivated neutral alumina (Woelm), covered by 0.5 cm anhydrous $NaSO_4$, in a microcolumn made from a diSPo transfer pipet, 5 mm i.d. by 140 mm (Scientific Products), (Law, 1970). The aldrin fraction was eluted with 2 ml of hexane. On further elution with 6 ml of hexane the dieldrin fraction was collected. The aldrin fraction was diluted 1:100 with hexane and analyzed in a double-beam, Perkin Elmer 124D spectrophotometer, between 195-250 nm, using a 1 cm quartz cell. The dieldrin fraction was analyzed, representing a 1:2 dilution of the original sample, in the same manner.

Cultures of filamentous organisms, including Z. xylestrix, were batch-extracted with twice their volume of chloroform during a 10 min disruption with a Branson S-75 sonifier at 20 kHz, to remove pesticides from the mycelial exterior. Analytical lindane was used as the internal standard to correct manipulation losses. Cultures were treated just prior to extraction with an amount of lindane identical to the concentration of cyclodiene involved. During disruption, the extraction vessel was immersed in an ice bath at 15 C. After disruption, the probe was rinsed with chloroform and the batch shaken for 15 min on the reciprocating shaker. Following separation, the aqueous phase was discarded, and the solvent phase washed and dried over anhydrous Na_2SO_4. A suitable volume (usually 10.0 ml)

was transferred to a glass-stoppered 50-ml Erlenmeyer flask, and evaporated to dryness at 60 C in a stream of N_2. The residue was dissolved in an equal volume of hexane and used for analysis without further purification. Non-filamentous organisms were extracted with 30 ml of isopropanol:hexane (1:2 vol/vol) (Mendel, 1967) to avoid the thick emulsion which formed when chloroform was the extractant. Otherwise, the extraction procedure was the same as for filamentous organisms.

When cells and cell-free media were extracted separately, the procedure employed was as follows: After stopping the interaction with 20% TCA, the whole culture was Millipore-filtered through a tared Whatman GF/A disc. The mycelial pad was washed twice with 50 ml of DW and once with 50 ml of 50% acetone. The entire filtrate was extracted for 1 hr in a 250-ml separatory funnel with 75 ml of the 1:2 mixture of isopropanol:hexane. The aqueous phase was discarded and the hexane phase washed, dried, and concentrated as required for the specific concentration of pesticide expected. The filter discs containing the cells were dried overnight in a desiccator, folded, and placed in a 25 by 80 mm Whatman paper extraction thimble. The mycelium was then extracted for 6-12 hr in a Soxhlet extractor with 200 ml of a 1:1 (vol/vol) mixture of hexane-acetone. The temperature of the heating plate was adjusted to produce approximately 12 siphon cycles/hr. After extraction, the solvent was evaporated to dryness in a Rinco rotary evaporator, and the residue dissolved in a minimal

amount of hexane.

Thin-layer chromatography. Plates were prepared in the laboratory according to the methods of Walker and Beroza (1963) and Official Methods of Analysis (1968). Double-strength window glass plates, 20 by 20 cm, of uniform width and thickness, were coated with a Research Specialties applicator set to provide a 250 um layer. The absorbents used and their weights in 100 ml of water were: Al_2O_3-G (Research Specialties) 60 g, Silica gel G (Research Specialties) 50 g, Silica gel H (Kensington) 50 g. The desiccated absorbent powder was placed in a 250-ml Erlenmeyer flask and the required amount of water added. After shaking moderately for 45 sec, the slurry was poured immediately into the applicator chamber; the coating was performed within 2 min. The plates were allowed to dry in position for 15 min and then finally dried in a forced draft oven for 30 min at 80 C. Plates of Al_2O_3-G were prewashed with 50% acetone and redried for 45 min at 80C. All plates were stored in a large, sealed jar containing Drierite.

Nine samples were spotted at 1 cm intervals on a line 4 cm from the bottom and 1.5 cm from each side, using either 10 or 20 ul disposable micropipettes. The preferred method of irrigation was to pour the solvent into the tank just prior to immersing the plates (Kovacs, 1965). The covers of the tanks were sealed with masking tape and irrigated to the required distance: 10 cm for Al_2O_3, 15 cm for Silica gel. Pre-coated 20 by 20 cm Mylar plates covered with a

250 um layer of Silica gel G (Eastman Kodak Type K30122) were activated for 30 min at 100 C. The irrigation systems used at different times were: heptane-acetone (49:1), ethyl ether-hexane (9:1), benzene-ethyl acetate (3:1), hexane-acetone (4:1), dichloromethane-carbon tetrachloride (1:1), and chloroform-methanol (1:9). Two dimensional thin layer plates were used to detect metabolic products in the aqueous phase after chloroform extraction. The solvent system used in these instances was chloroform-methanol-NH_4OH 17% (2:2:1) (Kirschner, 1967). Plates were sprayed with 0.25% ninhydrin or superimposed on high contrast x-ray film. Unless otherwise stated, migration rate values of labeled or unlabeled spots found in thin layer chromatography (TLC) preparations are given as relative to either aldrin (R_a=R_f unknown/R_f aldrin) or dieldrin (R_d=R_f unknown/R_f dieldrin).

Gas liquid chromatography. All GLC determinations, except those carried out at the Primate Research Laboratory, were performed in either a Perkin-Elmer Model 900 or a Varian Aerograph Model 2100 gas chromatograph, both equipped with FID and 0.31 by 180 cm glass columns packed with 3% OV-17 on Chromosorb WHP 80/100 mesh. The chromatographs were operated isothermally at 195 C with an injection port temperature of 225 C and a detector (manifold) temperature of 210 C. The carrier was pre-purified N_2 with a flow rate of 50 to 65 ml/min. Normally, all determinations were made with an attenuation of 4 in the 10^{-10} ampere range. The usual injection sample was 2 ul with a 1 ul solvent flush. Under these conditions, a 0.08 ug/ul

standard of aldrin gave a 70% deflection on the recorder chart moving at 1.25 cm/min. For trace analysis, a higher injection sample (3-5 ul) and lower attenuation (2-8 x 10^{-11}) was used. Peak height was employed to calculate concentration maxima generated by unknown samples as compared to those generated by standards.

Action of cultures on unlabeled pesticides. Organisms isolated on H media were subcultured in 10 ml of F4 medium, supplemented with either peptone or glucose, containing 100 ug/ml analytical aldrin or dieldrin. Each organism was grown statically in duplicate for 15 days at 30 C. Appropriate controls included uninoculated media containing pesticides and inoculated media without pesticides. Analytical aldrin or dieldrin was added in a minimal amount of acetone, initially. Growth was stopped by 0.1 ml of 20% TCA/10 ml of medium and the whole culture extracted immediately.

Duplicate or triplicate cultures of Z. xylestrix, in 250-ml conical culture flasks containing 50 ml of 1410 M Basal media, were used. The medium was supplemented with either 0.01 or 0.5% glucose, or was used without glucose, as required. Each culture was inoculated with the standard 2 mg mycelium 25 ml of medium and shaken at 25 C. Included, as above, were reagent and cell controls; growth was stopped as before.

Action of cultures on labeled pesticides. Cultivation of grouped cultures, H media isolates, and Z. xylestrix, with ^{14}C-labeled aldrin or dieldrin, followed the same

technique as for unlabeled pesticides. In all cases, the cultures contained either 0.019 uCi/ml ^{14}C-aldrin or 0.02 uCi/ml ^{14}C-dieldrin. Cultures were incubated statically in the dark at 30 C, except for Z. xylestrix which was grown at 25 C, in fluorescent light on the shaker. Controls included uninoculated media, with and without pesticides, and inoculated media to which pesticides were added just prior to extraction.

Cultures were extracted with twice their volume of chloroform and, after discarding the aqueous phase, a 10 ml sample of solvent fraction was concentrated to 0.2 ml (Matsumura and Boush, 1968). A 20 ul sample was spotted on different TLC adsorbents (Table 1). Irrigated plates were exposed to high contrast x-ray film (Fugi) for 25 days and developed in a Kodak Examat, automatic x-ray developer, model 2.

For Z. xylestrix, following chloroform extraction, 1 ml of aqueous phase was assayed in 10 ml of Aquascint II (International Chemical and Nuclear) using a Beckman CPM 100 scintillation counter. Similarly, 1 ml of the chloroform phase was counted in 10 ml of a solution containing 0.4 g of PPO (2,5-diphenyloxazole, p-terphenyl) and 5 mg of POPOP (1,4-bis-2,5-phenyloxazoly-benzene) in 100 ml of toluene. Quenching due to chloroform or precipitated salts from AcW was determined prior to analysis. The expected total activity originally added to each culture, in counts/min, was estimated from the specific activity, in uCi/mg, of the analytical labeled pesticide,

TABLE 1. Metabolite search protocol for Z. xylestrix extracts[a].

Method	TLC/$AgNO_3$						TLC/^{14}C-labeled							GLC
Support	Al_2O_3			Silica G			Al_2O_3	Silica G					Silica H	
Solvent	1	2	3	1	2	3	1	1	2	3	4	5	6	2	
Substrate: Aldrin	ab	b	b	cd	cd	b	a	a	a	a	a	a	a	a	bcd
Substrate: Aldrin-glucose	ab	b	b	cd	cd	b	a	a	a	a	a	a	a	a	bcd
Substrate: Dieldrin	ab	b	b	cd	cd	b	a	a	a	a	a	a	a	a	bcd
Substrate: Dieldrin-glucose	ab	b	b	cd	cd	b	a	a	a	a	a	a	a	a	bcd

[a]Extractants: (a) chloroform, (b) hexane, (c) ether, (d) acetone. Mobile solvents: (1) heptane-acetone, (2) ether-hexane, (3) benzene-ethylacetate, (4) hexane-acetone, (5) dichloromethane-carbontetrachloride, (6) chloroform-methanol.

rather than by directly counting activity of a suitable sample. Values, however, were corrected by control recoveries. Developed x-ray films were scanned also with a Photovolt Multiplier Photometer, Model 520-A, equipped with a search lamp (575 nm filter) scanning tray, and a linear/log, Varicord 43 recorder. Quantitation was achieved by determining peak area.

RESULTS

Conditions monitored at sampling stations and results obtained with isolation systems are summarized in Table 2. Strong currents were not observed during sampling except at station 05, and none of the local stations gave a visual appearance of heavy pollution. Salinity measurements showed stations 02, 04 and 08 to be brackish, while stations 03 and 05 were oceanic. Station 01, located 7.6 km inland was the only location to consist of fresh water. Temperature, pH, and dissolved O_2 data were within the expected ranges.

The composite sample from station 01 showed considerable concentrations of other organochlorine pesticides, especially DDT, DDD, and DDE. Remaining stations, however, failed to show detectable concentrations of chlorinated pesticides.

Of the 160 organisms isolated, 53% were isolated from surface water. Thirty-two organisms, isolated from the impregnated strips, are included since the canisters were designed to rest upright on the bottom or a few feet above. Identification per se was not attempted on primary isolation, but many organisms, particularly Streptomyces sp., were presumed owing to organoleptic manifestations e.g., strong earthy odor, etc. Although selective media were especially designed for actinomyces and filamentous fungi, other species were not excluded. All isolates were aerobes or facultative anaerobes.

TABLE 2. Isolation systems and stations.

Station	Location	Sample	Conditions[a]	System	Medium	Isolates[b] S	Isolates[b] W
01	Canal E3 Palm Bch Co, Fla.	3	Temp 22 %S 0.5 pH 7.2 O_2 75 Aldrin 0.1 Dieldrin 0.6	A	AC	14	17
					F	8	10
					F4	4	-
					Total	26	27
					Station	53	
03	Boca Inlet Boca Raton Fla	1	Temp 27 %S 3.5 pH 8.1 O_2 64 Aldrin 0.1 Dieldrin 0.1	A	AC	-	-
					F	1	-
					F4	4	-
					Total	5	-
					Station	5	
SB	St. Barbara Canal, Calf.	4	Temp - %S - pH - O_2 - Aldrin - Dieldrin -	A	AC	8	3
					F	1	-
					F4	9	2
					H	1	-
					Total	19	5
					Station	24	
04	Ocean Ridge Inlet Boyton Bch Fla	1	Temp 32 %S 2.5 pH 8.0 O_2 76 Aldrin 0.1 Dieldrin 0.1	B	AC	5	4
					F	-	-
					F4	2	4
					H	-	2
					Total	7	10
					Station	17	
02	ICWW Boca Raton Fla	2	Temp 27 %S 1.5 pH 7.5 O_2 62 Aldrin 0.1 Dieldrin 0.1	A	AC	-	3
					F	3	1
					F4	-	-
					H	-	2
					Total	3	6
				B	AC	-	-
					F	-	-
					F4	10	4
					H	5	1
					Total	15	5
					Station	29	
08	ICCW Boca Raton Fla	1	Temp 30 %S 2.5 pH 8.1 O_2 29 Aldrin - Dieldrin -	C	AC	-	5
					F	-	3
					F4	-	10
					H	-	1
					Station	19	
05	Pompano outfall boil	1	Temp 29 %S 3.5 pH 8.2 O_2 97 Aldrin 0.1 Dieldrin 0.1	C	AC	-	3
					F	-	4
					F4	-	4
					H	-	2
					Station	13	

[a] O_2: ppm; aldrin, dieldrin in ppb; %S: salinity.

[b] S: sediment, W: surface water.

Table 3 indicates the number of organisms in each deliberately mixed culture group, isolation station, and the morphological types involved. Of 129 organisms screened in this manner, only 10 grew in H media after three successive transfers bringing to 24 the total number of organisms isolated on H medium either during isolation or after adaptation from grouped cultures.

The same culture groups, excluding those isolates growing on H media, were screened in media containing either ^{14}C-labeled aldrin or dieldrin. A radioautogram of thin-layer plates of ^{14}C-aldrin extracts from the grouped cultures is shown in Fig. 5. Three patterns suggesting possible metabolites are recognizable. Groups VI, VIIa and IX gave spot C (R_a=0.23) which was not produced by the controls or any other group. Spot B (R_a=0.17) was produced by most groups with the exception of VII, VIII, and the controls. Spot D, not manifested by groups VII or VIII, but produced by the controls, might have been an artifact. Spot F and G, dieldrin and aldrin respectively, arose from the original labeled-pesticide vial. A similar presentation for ^{14}C-dieldrin is given in Fig. 6; again, possible metabolites are indicated. Group V, filamentous organisms, were the most active yielding four well-separated spots not shown by the controls. Spot C, in Fig. 6 for dieldrin (R_d=0.25, R_a=0.2), may have been identical with spot C in Fig. 5. Spot F probably represented a contaminant, corrected for by the controls, and spot G represented dieldrin. Groups 043ST, a mixed natural culture from

TABLE 3. Isolates from mixed culture groups on "H" media.

Group	No. of organisms	Station	Type of organism [a]	No. isolated
I	9	01	NF	None
II	10	01	NF	None
III	10	01	NF	1
IV	8	01	F	2
V	7	01	F	None
VI	10	SB	F	None
VII	8 2	02 SB	NF	1
VIII	3 4	02 03	NF	None
VIIIa	11	02	F	None
IX	9	SB	NF	1
X	10	04	NF	1
XI	8	05	NF	1
XIa	10	08	NF	1
XII	2 8	05 08	F	2

[a] NF: non-filamentous; F: filamentous

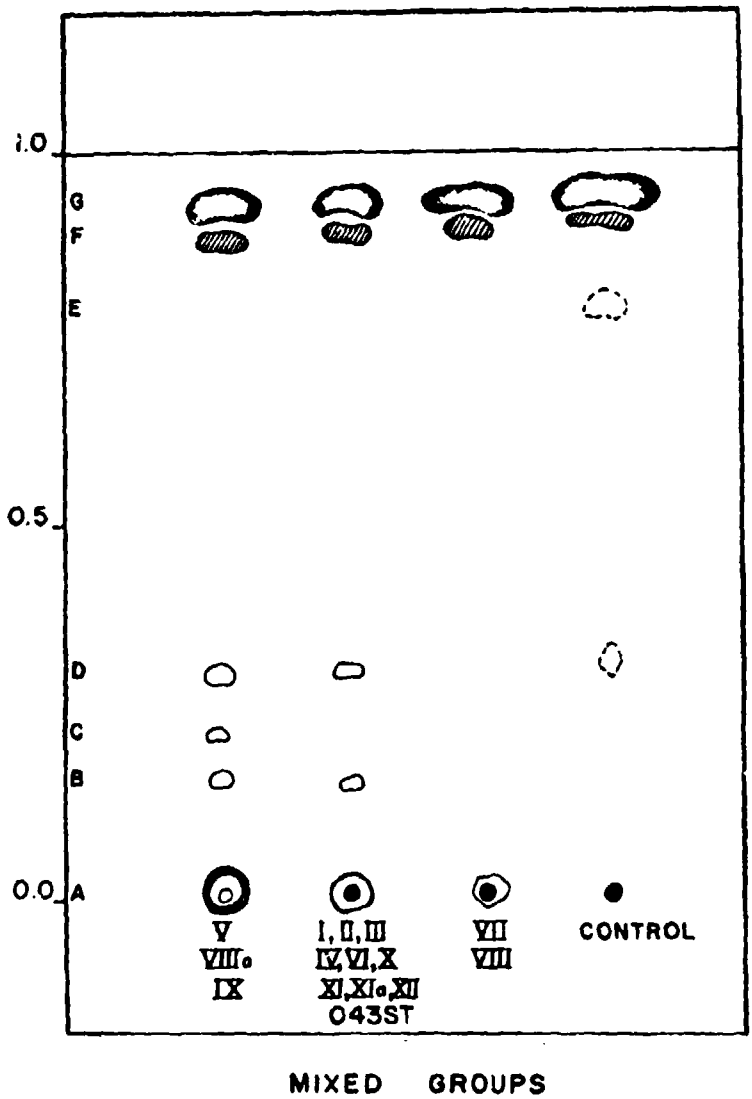


FIG. 5. Thin-layer radioautogram of ^{14}C-aldrin mixed group culture extracts; 20 ul fractions irrigated 15 cm from origin on Silica gel G (hexane-acetone, 4:1). X-ray film exposure time, 26 days. Shaded, barred, closed, and dotted circles, in that order, represent an arbitrary scale of decreasing intensities of radioautographic spots.

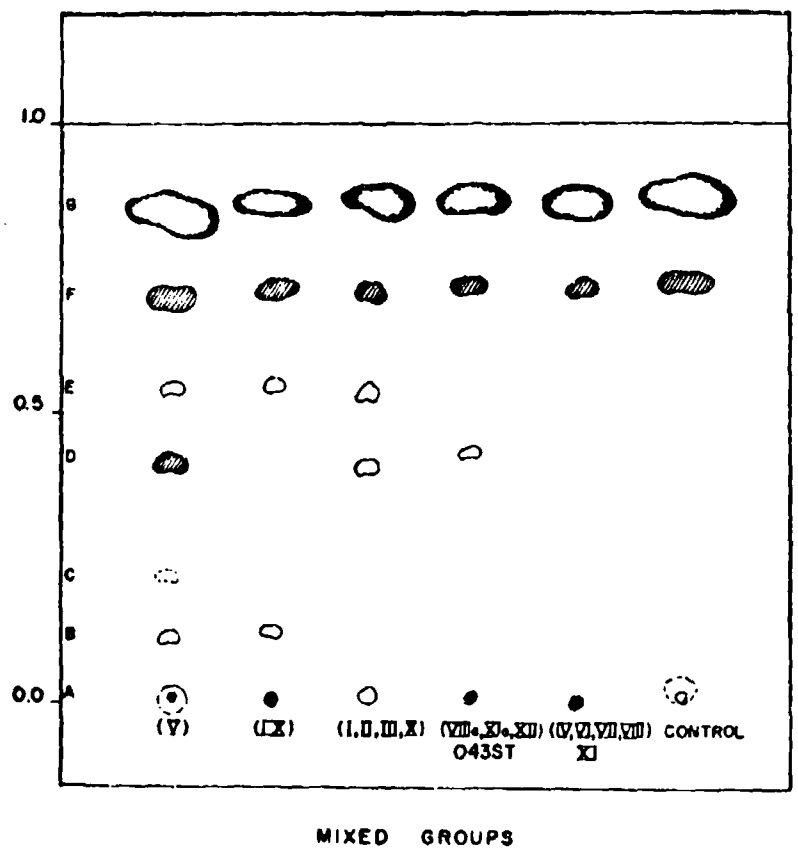


FIG. 6. Thin-layer radioautogram of ^{14}C-dieldrin mixed group culture extracts; 20 ul fractions irrigated 15 cm from origin on Silica gel G (hexane-acetone, 4:1). X-ray film exposure time, 26 days. Shaded, barred, closed, and dotted circles, in that order, represent an arbitrary scale of decreasing intensities of radioautographic spots.

station 04, System B, was obtained 20 weeks after the last addition of pesticides. Other spots gave R_a or R_d values similar to those reported elsewhere (Matsumura et al., 1968) for aldrin metabolites, but none were chemically characterized.

Of the organisms isolated on H medium, ten were discarded as duplicates. On TLC screening of whole F4 culture extracts of the remaining strains, $AgNO_3$ reagent failed to indicate metabolites.

An outline of major cultural characteristics of H media isolates, kept for further study, is shown in Table 4. Growth on H media is indicated as "direct" if the organism was isolated directly from the field station samples or "adapted" if the organism was isolated on H media from one of the grouped cultures. Only three of these isolates were filamentous organisms: strain 1145, 8242, and 8247 (Fig 7). Strains 1145 and 8247 appeared to be Trichoderma sp.

Most other isolates were Gram negative rods or coccobacillary forms (Fig 7) which appeared to attack sugars oxidatively and were cytochrome oxidase-positive. Flagella were demonstrated conclusively only with isolate 043S1. Isolate 22S30 showed polar flagellation when first isolated, but after several transfers flagellation could not be demonstrated. This was true also on later observation of the original stock culture. Isolates 1130 and 2230 produced a water soluble green pigment in both experimental and transfer liquid media, but not in nutrient agar. Strain

TABLE 4. Cell and cultural characteristics of H-media isolates.

Isolate	Station	Growth on "H"	Colony morphology	Cell morphology	Flagella
1130	01	Adapted	Circular 2 mm smooth, glistening pale yellow.	Gram negative rods, pleomorphic, long chains.	None
0SB1	SB	Direct	Punctiform, smooth, glistening, light.	Gram variable 1-2 um rods, palisades.	None
022S	02	Direct	Irregular, 1 mm smooth translucent.	Gram negative slender, long chains.	None
2230	02	Adapted	Irregular, 2 mm rugose, translucent.	Gram negative slender, 2 um curved rods.	None
043S1	04	Direct	Punctiform, smooth, translucent.	Gram negative coccobacilli 1.5-2.5 um	1-? Polar
22S30	02	Adapted	Confluent mucoid, glistening, cream.	Gram negative 0.5-1 um curved rods.	0-1 Polar
053M-2	05	Direct	Circular 1 mm smooth, glistening white.	Gram negative slender, curved, rosettes.	None
082S	08	Direct	Confluent, mucoid circular, glistening.	Gram negative 0.5-1 um coccobacilli.	None
SB30	SB	Adapted	Circular 1 mm smooth, translucent.	Gram negative 1-2 um rods, small chains.	None
4330	04	Adapted	Punctiform, smooth, glistening, gray.	Gram negative coccobacilli 2-5 um	None
8230	08	Adapted	Confluent, mucoid, circular, glistening, cream.	Gram negative 1 um curved & coccoid rods.	None
1145	01	Adapted	Rapid, green aerial mycelia.	Hyaline septate, branched, one-celled ovoid conidiophores.	NA
8242	08	Adapted	Powdery, white mycelia.	Hyaline, septate one-celled cylindrical conidia (Arthrospores).	NA

TABLE 4. (continued)

Isolate	OF media[a]						Growth on									Tentative identification
	Glucose O	Glucose F	Sucrose O	Sucrose F	Lactose O	Lactose F	Gelatinase	Oxidase	Catalase	H-basal	Tween 80	Ethanol	Acetone	Aldrin	Dieldrin	
1130	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	Flavobacterium
OSB1	+	+	+	+	+	+	-	±	+	-	+	-	-	-	-	Arthrobacter
022S	+	-	+	-	+	+	-	+	+	-	+	-	±	-	-	Achromobacter
2230	+	-	+	-	+	-	-	±	+	-	+	+	+	-	-	Achromobacter
043S1	+	+	+	-	+	-	+	+	+	-	+	-	-	-	-	Vibrio
22S30	-	-	-	-	-	-	+	+	+	-	+	+	-	-	-	Vibrio
053M2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	Non-fermentative rod
082S	-	-	-	-	-	-	+	+	+	±	+	±	±	-	-	Alcaligenes
SB30	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	Non-fermentative rod
4330	-	-	-	-	-	-	+	-	±	-	+	±	-	-	-	Non-fermentative rod
8230	-	-	-	-	-	-	+	+	+	-	+	+	+	-	-	Alcaligenes
1145	NA									-	+	-	-	-	-	Trichoderma
8242	NA									-	+	-	-	-	-	Monocillium

[a] OF media (oxidation-fermentation): O = oxidation, F = fermentation
NA not applicable.

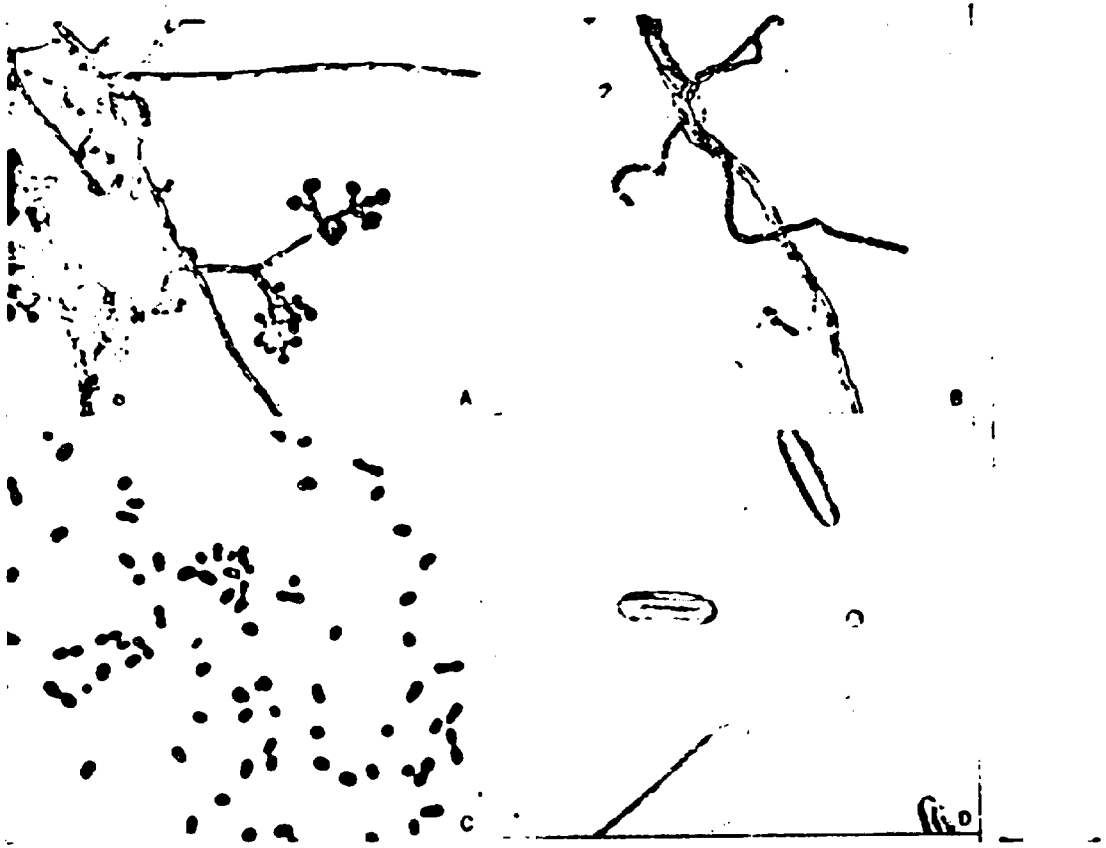


FIG. 7. Representative H media isolates and laboratory ecosystem microflora: (A) isolate 1145 (x 200), (B) isolate 8242 (x 200), (C) isolate 2230 (x 900), (D) wet mount preparation from system B, station 04, 98 days old (x 400).

OSB1, when isolated, was consistently Gram positive in young nutrient agar cultures, but on repeated transfer became Gram variable to negative. None of these isolates was able to grow in the H medium basal alone or with aldrin or dieldrin as sole carbon sources, but all grew on H media containing 0.05% Tween 80. Although organisms isolated from brackish or littoral waters were cultured repeatedly in 75% ASW media, none was shown to require ASW for growth.

Selected H media isolates were tested for action on pesticides using GLC quantitation. Table 5 shows the pesticide recoveries after possible cometabolism with glucose and peptone. Most recoveries were greater than 90% which equalled those for the controls. Biodegradation products were not detected by FID.

When all 14 H media isolates were tested for pesticide degradation, using ^{14}C-labeled aldrin or dieldrin, six showed profiles with aldrin and/or dieldrin which differed from the controls. Possible conversion products found in radioautograms (Silica gel G TLC) of ^{14}C-aldrin extracts from H media isolates are shown in Fig 8. Spots H and G showed aldrin and dieldrin, respectively. Spot F (R_a=0.57), only from isolate 2230, was shown faintly. Other R_a values were: E: 0.26; D: 0.19; C: 0.11, and B: 0.07. Spot B, from the extracts of culture 8247, may have been an artifact, produced by TCA treatment, present in all cultures and controls. Fig 9 presents the results obtained in cultures grown with ^{14}C-labeled dieldrin. Only spot C, shown by extracts of all six isolates (R_d=0.37), and spot B, found

TABLE 5. Action of H-medium isolates on 100 ug pesticide/ml (GLC determinations).

Organism	Medium[a]	Days	Percent aldrin[b] recovered	Percent dieldrin[b] recovered
1130	F4(DW)SP	15	92.0	103.0
2230	F4(ASW)SP	20	88.0	97.0
0SB1	F4(ASW)SP	20	96.6	91.0
022S	F4(ASW)SP	20	89.9	91.3
1145	F4(DW)SG	15	107.0	96.0
8242	F4(ASW)SG	15	95.0	105.0
8247	F4(ASW)SG	15	108.0	90.0

[a] SP: supplemented with peptone; SG: supplemented with glucose.

[b] Values adjusted on internal standard.

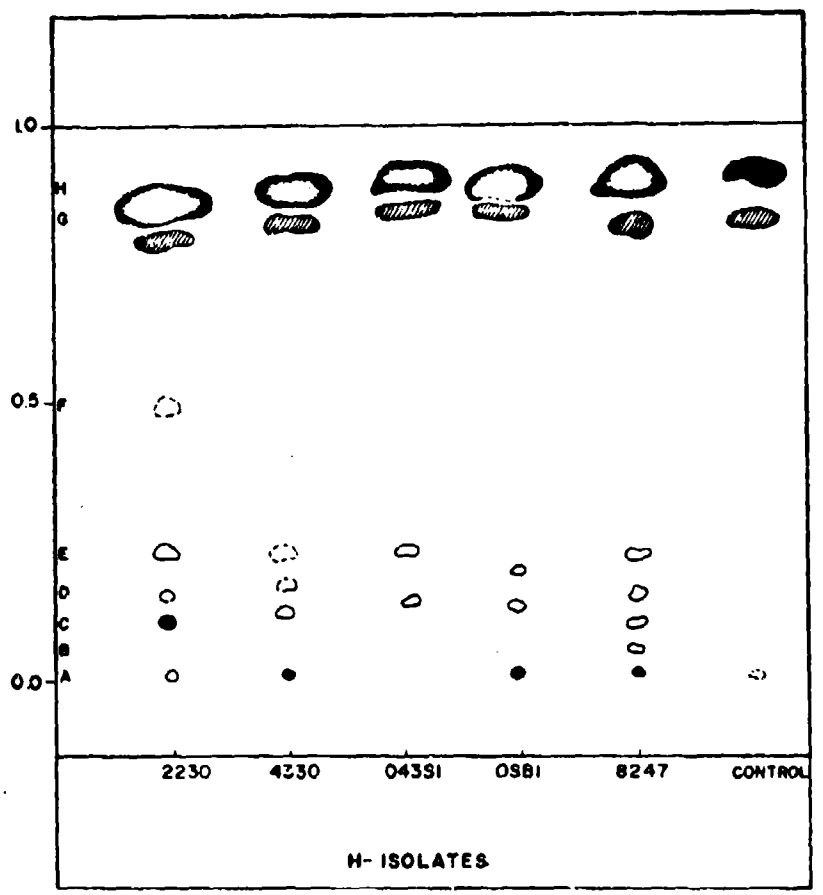


FIG. 8. Radioautogram of ^{14}C-aldrin H-medium isolate extracts; 20 ul fractions irrigated 15 cm from origin on Silica gel G (hexane-acetone, 4:1). X-ray film exposure time, 30 days. Shaded, barred, closed, and dotted circles, in that order, represent an arbitrary scale of decreasing intensities of radioautographic spots.

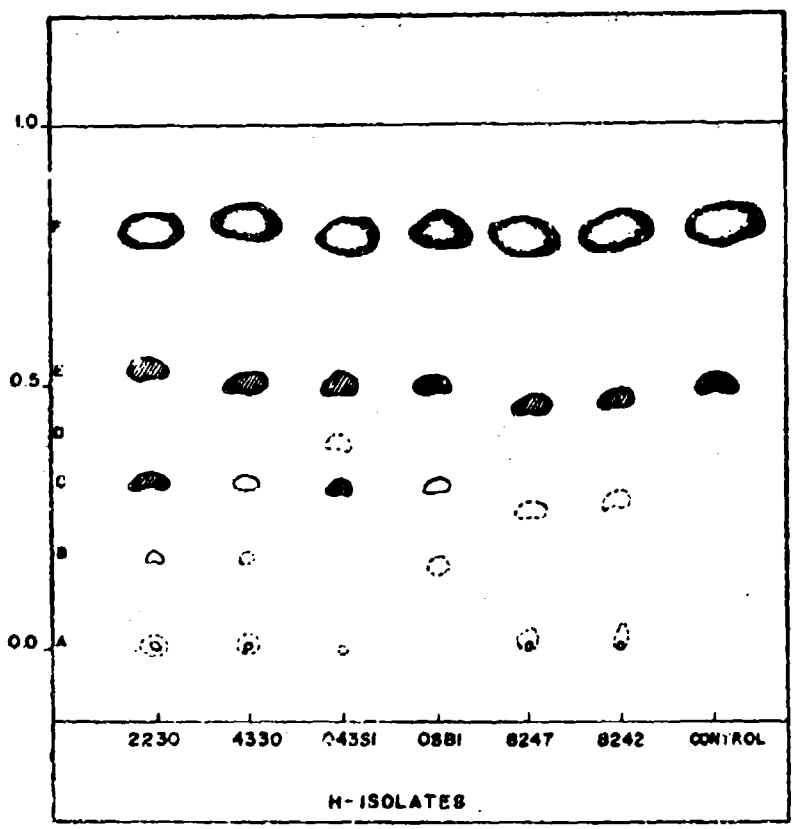


FIG. 9. Radioautogram of ^{14}C-dieldrin H-medium isolate extracts; 20 ul fractions irrigated 15 cm from origin on Silica gel G (hexane-acetone, 4:1). X-ray film exposure time, 30 days. Shaded, barred, closed, and dotted circles, in that order, represent an arbitrary scale of decreasing intensities of radioautographic spots.

in isolates 2230, 4330, and OSB1, appeared to be significant. Spot B appeared to be identical to spot E in the aldrin chromatograms and very similar to spot C in aldrin and dieldrin autoradiograms of the grouped mixed cultures. Spot F corresponded to dieldrin.

At the time of collection, and for the first 2-3 weeks, the surface water from System B, Station 04 remained turbid and the sediment brownish. Between 3 and 6 weeks, a strong H_2S odor emanated from the carboy and the sediment turned black. At the same time, the surface water cleared. After 6 weeks, the sediment began to turn gray while the surface water became green. A green scum also appeared on the carboy walls from the bottom up to within 5-7.5 cm of the surface. A trichome-forming organism along with numerous large and small rods was observed in wet mount preparations of the scum. At 12 weeks, the scum turned yellowish, and at 16 weeks the green had disappeared. Wet mount preparations during this time demonstrated only some protozoans, possibly Paramecium sp. At 20 weeks, the green coloration reappeared and characteristic diatomaceous forms, closely associated with what appeared to be Oscillatoria sp. (Fig. 7) were seen in wet mounts prepared from long filaments growing on the carboy walls.

The Oscillatoria sp. and the diatom were grown in artificial media, but only the former could be grown in axenic culture (W. Thomann, unpublished data). Fig. 10 shows the results obtained by GLC and UV analyses of samples collected from System B, Station 04. Both methods demonstrated

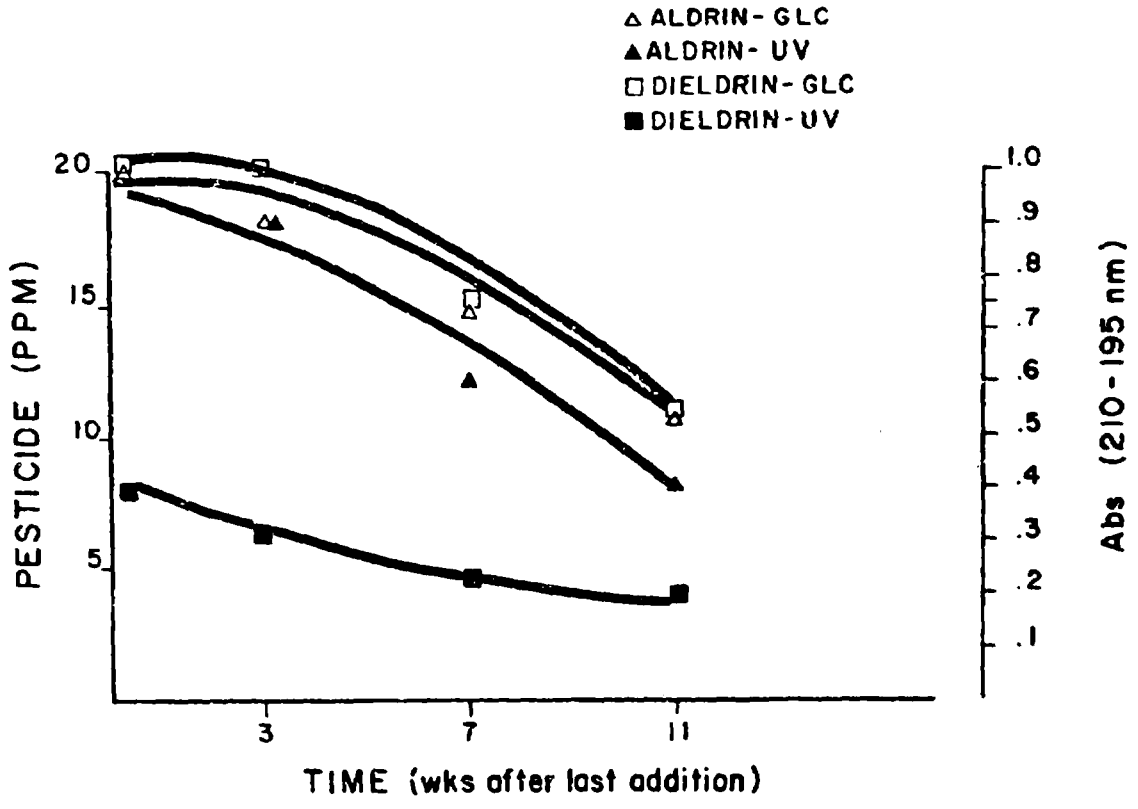


FIG. 10. Fate of 20 ppm (400 mg of each pesticide in 10 kg and 10 liters of sediment and water, respectively) of aldrin and dieldrin in system B, station 04 as determined by GLC quantitation and UV absorption.

a 50 and 60% reduction of aldrin and dieldrin, respectively. The decline was progressive rather than abrupt, and pesticide metabolites were undetectable.

Growth of the cellulolytic marine fungus Z. xylestrix, at different aldrin or dieldrin concentrations in 1410 M Basal media, is exemplified in Fig. 11. Mycelial increase, at 10 and 100 ug/ml of pesticide was comparable to that obtained at the same concentrations of glucose. Growth declined above 250 ug pesticide/ml and was very sparse at 750 and 1000 ug/ml. In a time-course experiment, maximal growth occurred at 8 days (Fig. 12). At this time, cultures containing 100 ug/ml of aldrin or dieldrin gave higher mycelial yields than cultures with the same glucose concentration.

When the effect of serially transferring the fungus in pesticide media became of interest, growth responses were observed using inocula from cultures exposed and unexposed to pesticide. Growth responses obtained from all inocula showed higher yields of mycelium in pesticide media than in glucose media (Fig. 13). Aldrin supported the highest yields at 10 days and aldrin-grown inocula gave the highest yields of all in aldrin media.

Further characterization of the growth of Z. xylestrix, in the presence of aldrin or dieldrin, by dry wt and cell N determinations, showed higher yields by both methods (Fig. 14). A similar pattern was observed when the basal medium was supplemented with 100 ug glucose/ml (Fig. 15). In both cases, maximal growth occurred at 10 days.

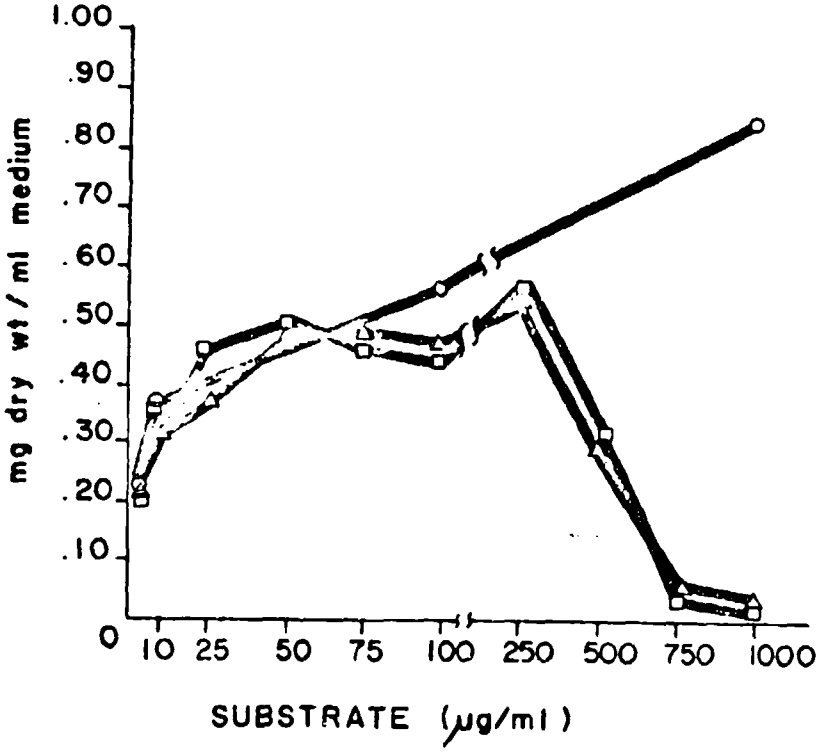


FIG. 11. Growth responses of Z. xylestrix, at different concentrations of aldrin (Δ), dieldrin (□), and glucose (○) in 1410 M Basal medium. Cultures were shaken for 10 days at 25 C.

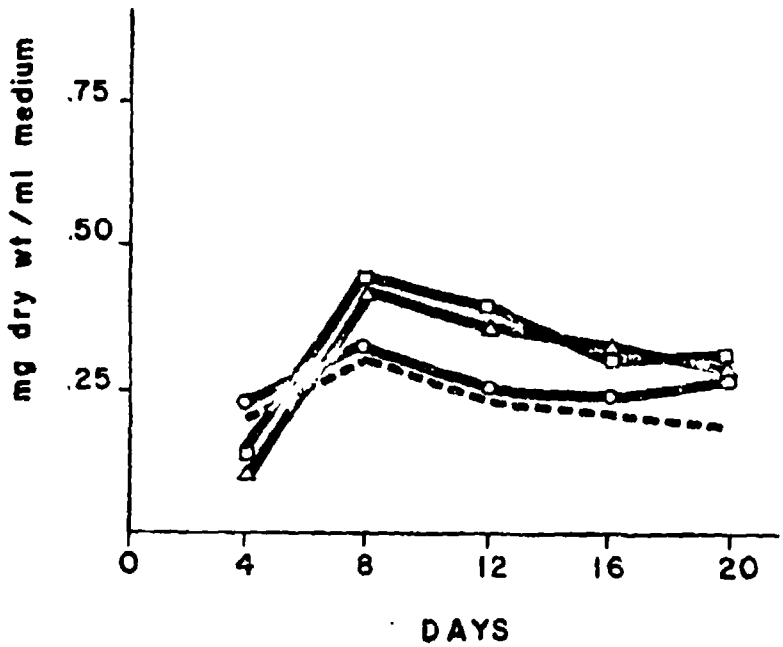


FIG. 12. Growth profile of Z. xylestrix in 1410 M Basal medium containing 100 ug/ml of either aldrin (Δ), dieldrin (□) or glucose (○). Dotted line represents 1410 M Basal culture without substrate.

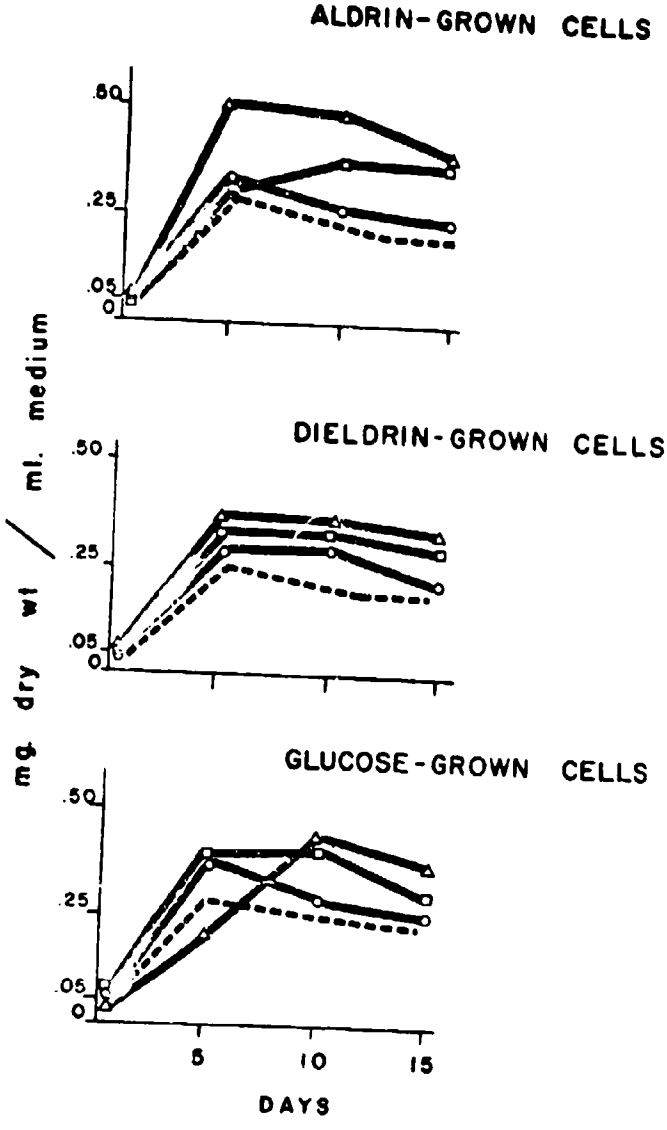


FIG. 13. Growth profiles of Z. xylostrix previously grown in either aldrin, dieldrin, or glucose, in 1:10 M Basal medium containing 100 ug/ml of aldrin (Δ), dieldrin (□) or glucose (O). Dotted line represents 1:10 M Basal culture without substrate.

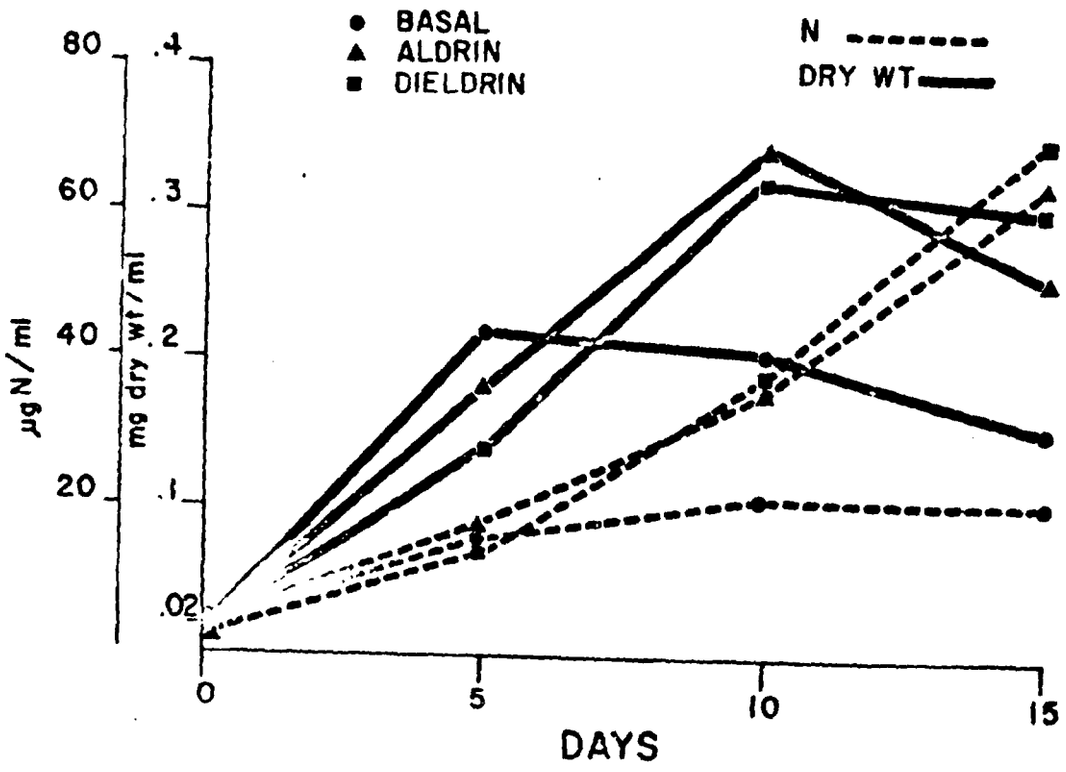


FIG. 14. Growth profiles correlating mycelial dry wt and cell N of Z. xylestrix in 1410 M Basal medium containing 100 ug/ml of the substrate.

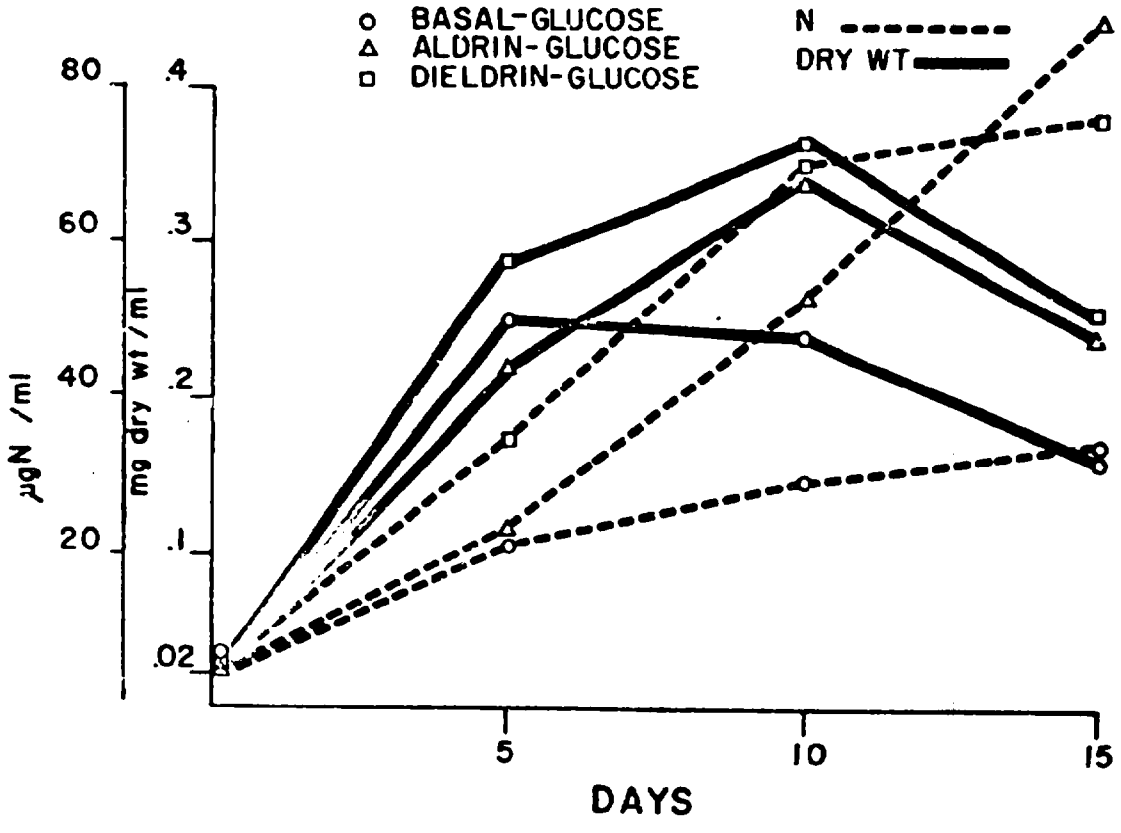


FIG. 15. Growth profiles of Z. xylestrix, correlating mycelial dry wt and cell N in 1410 M Basal medium containing 100 ug/ml of glucose and individual pesticides as cosubstrates.

The fate of pesticide in chloroform-extracted, whole cultures of Z. xylestrix, as determined by GLC, is shown in Table 6, where 15 day incubation recoveries are listed along with extraction method. Wet mount preparations indicated 70-90% mycelial disruption after insonation for 10 min. Recoveries of 85-87% were obtained against lindane and values corrected. Inoculum carry-over, however, averaged less than 1 ug/ml.

The results of time-course experiments on aldrin and dieldrin uptake by Z. xylestrix are shown in Fig. 16 and 17. As shown in the former, glucose made no significant difference in aldrin recovery. Uptake appeared to level-off at 10 days. This coincided with the age at which maximal growth was obtained with dieldrin (Fig. 17). Values, corrected for recoveries in the controls without internal standard, were aldrin 90% and dieldrin 93%. Extracts were extensively analyzed by TLC (Table 1); however, no metabolites were detected with $AgNO_3$ in any chromatographic system. Trace analyses by GLC demonstrated one peak with a retention time, relative to aldrin (RRT_a), of 1.12 min in most aldrin and aldrin-glucose cultures. This did not occur in the controls (Fig. 18). Trace analyses (GLC) of dieldrin and dieldrin-glucose similarly demonstrated a small shoulder just off the solvent front, with a retention time relative to dieldrin (RRT_d) of 0.402. This also was not found in control cultures (Fig. 19).

Activity distribution between aqueous and solvent phases in extracts of Z. xylestrix cultures, with and

TABLE 6. Pesticide uptake by Z. xylestrix.

Substrate	Culture volume (ml)	Extraction method	Initial pesticide (ug/ml)	Final pesticide (ug/ml)	Percent lost from medium
Aldrin	25	Chloroform shake	80	53.6	33
Aldrin-glucose	25	Chloroform shake	80	56.0	30
Aldrin	50	Chloroform insonation	80	60.8	24
Aldrin-glucose	50	Chloroform insonation	80	62.4	22
Dieldrin	25	Chloroform shake	80	48.0	40
Dieldrin-glucose	25	Chloroform shake	80	50.4	37
Dieldrin	50	Chloroform insonation	80	56.0	30
Dieldrin-glucose	50	Chloroform insonation	80	52.0	35

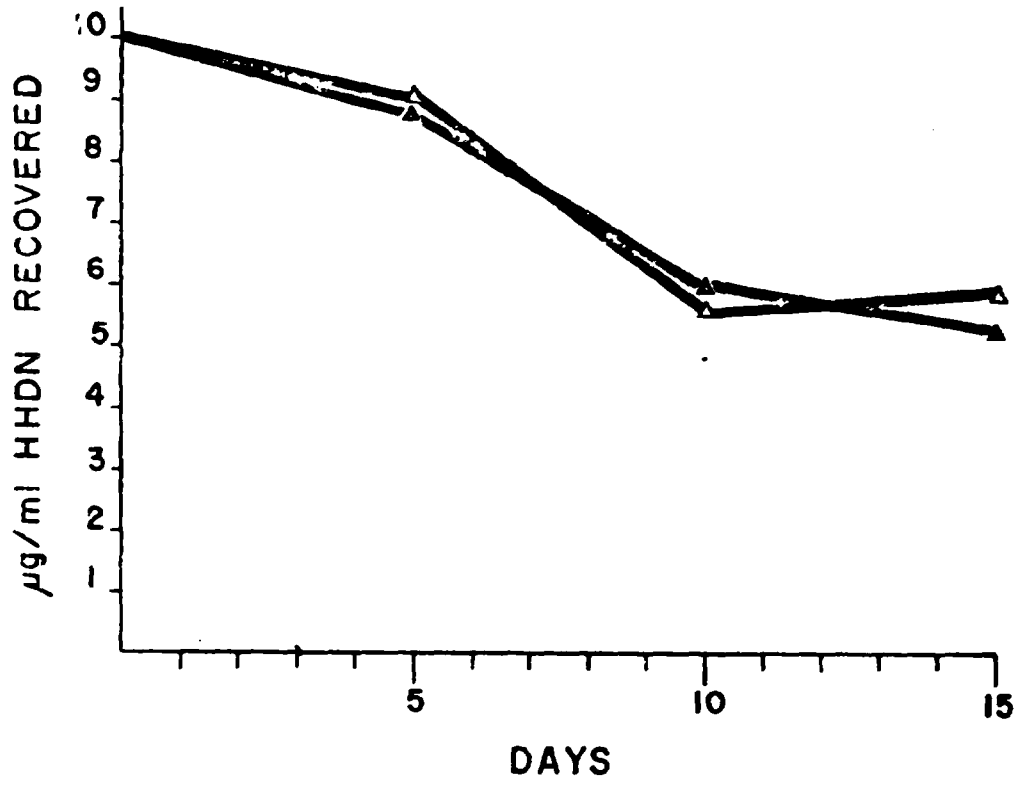


FIG. 16. Time-course of aldrin (99.5% HHDN) uptake by Z. xylestrix in 1410 M Basal medium and in the same medium supplemented with 0.5% glucose. Aldrin (Δ), aldrin-glucose (▲) (GLC quantitation).

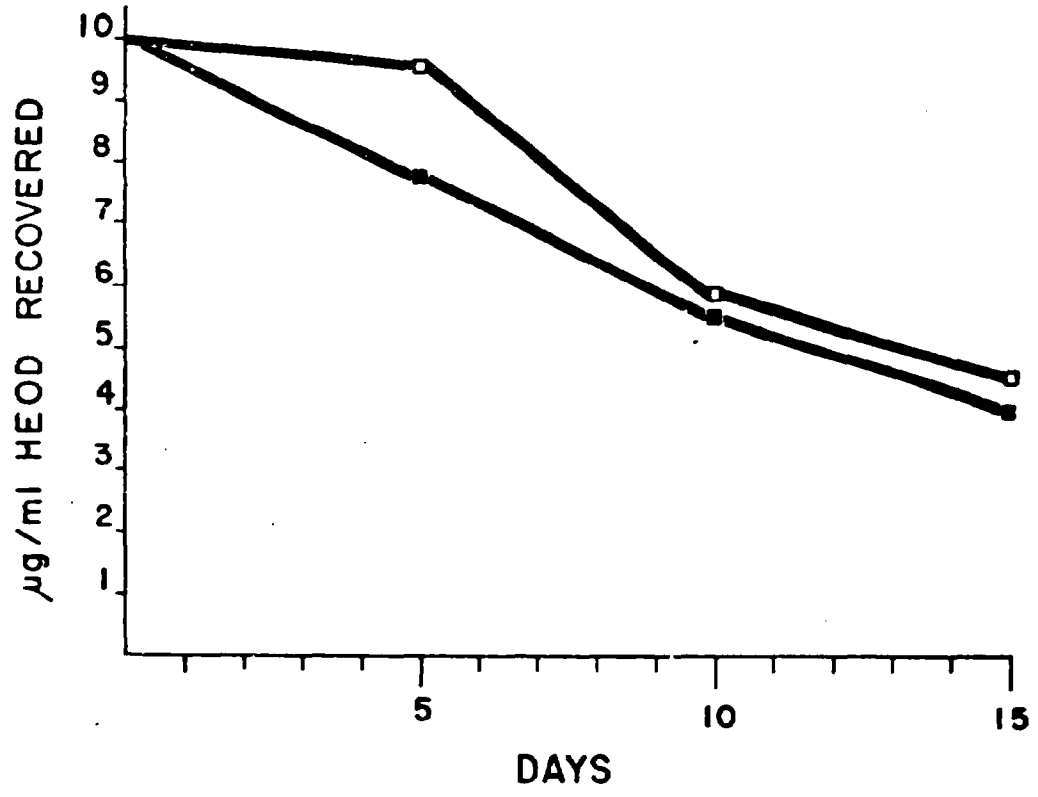


FIG. 17. Time-course of dieldrin (99.6% HEOD) uptake by Z. xylestrix in 1410 M Basal medium and in the same medium supplemented with 0.5% glucose. Dieldrin (□), dieldrin-glucose (■) (GLC quantitation).

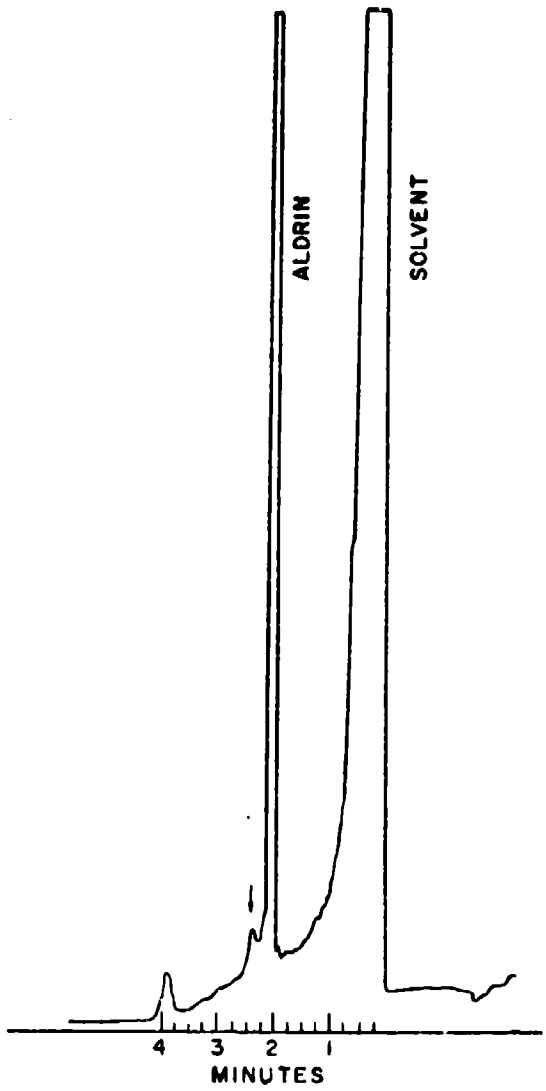


FIG. 18. Representative GLC/FID chromatogram of extracts of a 15 day old Z. xylestrix culture containing aldrin. Injection size: 3 ul; support: 3% SE-30 on Varaport 30, 100/120; attenuation: 2 x 10^{-1}. Arrow indicates peak not found in control preparations.

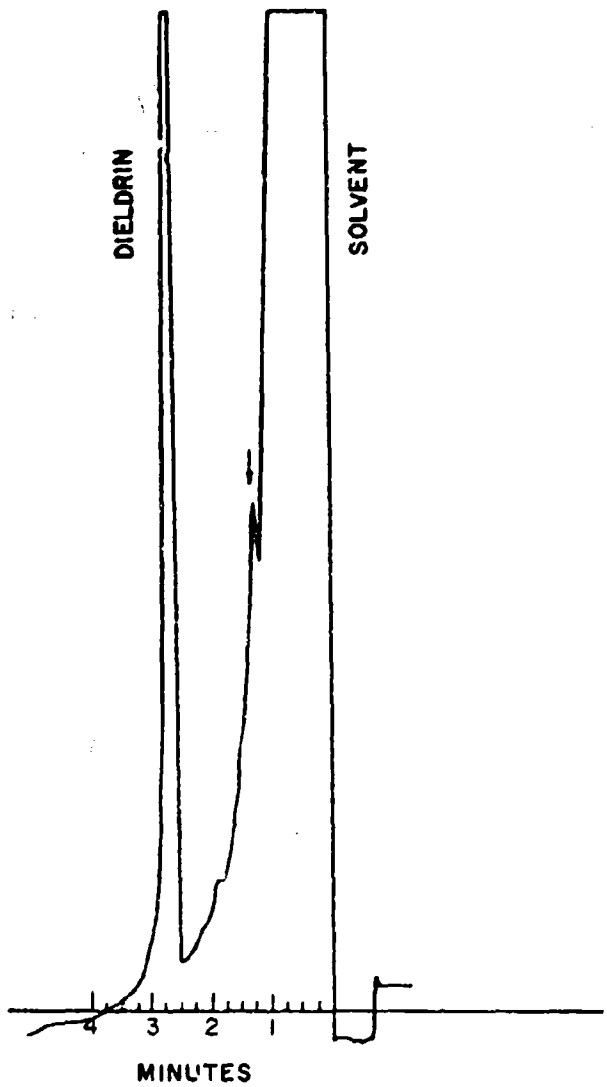


FIG. 19. Representative GLC/FID chromatogram of extracts of a 15 day old X. xylestrix culture containing dieldrin. Injection size: 5 ul; support: 3% SE-30 on Varaport 30, 100/120; attenuation: 8 x 10^{-11}. Arrow indicates shoulder not found in control preparations.

without glucose, is shown in Table 7. Corrected recovery values obtained for both pesticides represent the average of six determinations for aldrin and nine for dieldrin. The control recovery was 98% with negligible activity in the aqueous phase. In most cases, different determinations were within 5% of the mean. Neither radio-labeled nor ninhydrin-positive compounds were detected by subsequent TLC analyses of aqueous phases. Solvent phase TLC analyses, on the other hand, demonstrated the presence of a number of spots not present in the controls. The best separation of possible metabolites was obtained in hexane-acetone (4:1) (Fig. 20) and hexane-ethyl acetate (3:1) (Fig. 21). The R_a values achieved with hexane-acetone follow: spot F: 0.763, spot E: 0.322, spot D: 0.233, spot C: 0.136, spot B: 0.104. Artifacts were found in all cultures, as well as in the controls (spot A). Table 8 summarizes the results of scanning radiochromatograms irrigated with ether:hexane on Silica gel G. Although the spot intensities are not direct measurements of activity, they suggest the relationship between parent compounds and possible metabolites.

When washed mycelia and cell-free media were extracted separately, a considerable amount of the initial pesticide was found in the cells (Table 9). Values obtained at different pesticide and glucose concentrations represent the average of uncorrected duplicates. The overall recovery in the controls was 99% for aldrin and 92% for dieldrin. Of the initial aldrin concentration (50 ug/ml), 87% was found in the medium and 12% in the cells; 85% and

TABLE 7. Carbon-labeled pesticide activity distribution in Z. xylestrix extracts, as percent total activity.

Extraction phase	Substrate			
	Aldrin	Aldrin-glucose	Dieldrin	Dieldrin-glucose
Aqueous	3.0	3.4	0.18	0.5
Solvent	64.0	62.0	79.0	80.0

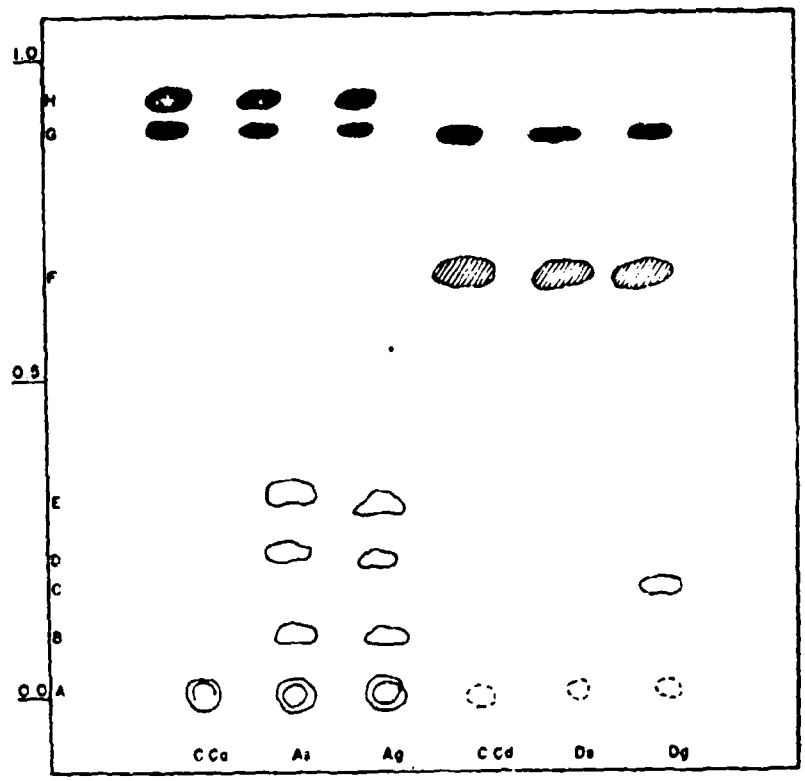


FIG. 20. Radioautogram of ^{14}C-aldrin and dieldrin extracts of Z. xylestrix on Silica gel G (hexane-acetone, 4:1). CCa: control cell preparation for aldrin, As: aldrin culture, Ag: aldrin-glucose culture; CCd: control cell preparation for dieldrin, Ds: dieldrin culture, Dg: dieldrin-glucose culture. Spot designator letters on ordinate.

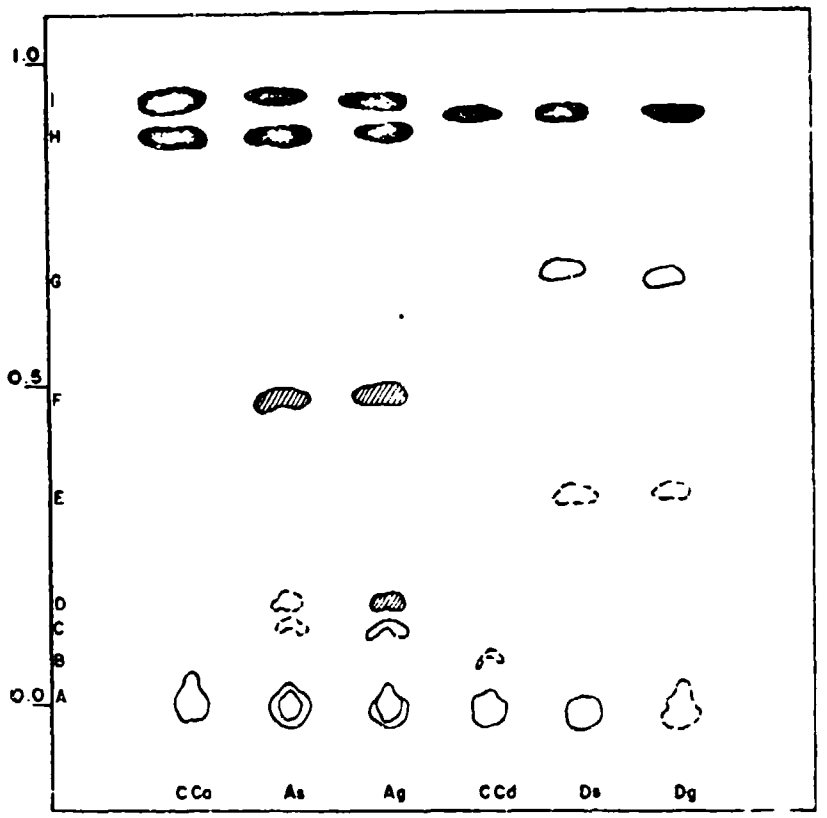


FIG. 21. Radioautogram of ^{14}C-aldrin and dieldrin extracts of Z. xylostrix on silica gel G (benzene-ethylacetate, 5:1). CCa: control cell preparation for aldrin, As: aldrin culture, Ag: aldrin-glucose culture: CCd: control cell preparation for dieldrin, Ds: dieldrin culture, Dg: dieldrin-glucose culture. Spot designator letters on ordinate.

TABLE 8. Radioautogram scanning data from Z. xylestrix extracts.

Culture	Spot[a]	R_f	Percent activity	Identity
Aldrin-control	A	0.90	92	Aldrin
	D	0.18	1	-
	E	0.06	6	-
Aldrin	A	0.91	82	Aldrin
	B	0.60	6	-
	C	0.35	3	-
	D	0.20	2	-
	E	0.04	6	-
Aldrin-glucose	A	0.92	75	Aldrin
	B	0.63	5	-
	C	0.39	7	-
	D	0.20	3	-
	E	0.04	8	-
Dieldrin-control	A	0.89	79	Dieldrin
	B	0.78	20	-
Dieldrin	A	0.89	71	Dieldrin
	B	0.81	23	-
	C	0.73	4	-
Dieldrin-glucose	A	0.89	82	Dieldrin
	B	0.81	13	-
	C	0.74	4	-

[a] Separation obtained on Silica gel G with ether-hexane as mobile phase.

TABLE 9. Distribution of aldrin and dieldrin between cells and media after action of *Z. xylestrix*[a].

Culture substrate	Initial pesticide (ug/ml)	Mycelial dry wt (g)	Pesticide in cells Total (ug)	ug/g	Percent	Pesticide in media Total (ug)	ug/ml	Percent	Pesticide recovered (%)
	10	0.020	195	9750	39	235	4.5	45	84
Aldrin	50	0.028	800	28571	32	-	-	-	-
	100	0.035	2500	71428	50	1500	30.0	30	80
	10	0.018	190	10555	38	260	5.2	52	90
Aldrin-glucose(0.01%)	50	0.029	975	30172	35	1250	25.0	50	85
	100	0.036	2750	76380	55	1600	32.0	32	87
	10	-	366	-	73	120	2.4	24	97
Aldrin-glucose(0.5%)	50	-	-	-	-	-	-	-	-
	100	-	3610	-	72	1050	21.0	21	93
	10	0.012	110	9166	22	385	77.0	77	89
Dieldrin	50	0.025	925	37000	37	-	-	-	-
	100	0.037	2800	75675	56	1300	26.0	26	82
	10	0.019	143	7526	28	270	5.4	54	82
Dieldrin-glucose(0.01%)	50	0.039	700	17948	28	1400	28.0	56	84
	100	0.039	3380	86666	67	650	13.0	13	80
	10	-	155	-	31	215	4.3	43	74
Dieldrin-glucose(0.5%)	50	-	-	-	-	-	-	-	-
	100	-	3190	-	63	750	1.5	15	78

[a] Cell-free media and washed mycelia extracted separately.

7% was the medium/cell distribution for dieldrin. Inoculum carry-over was 0.15 ug/ml for aldrin and 0.12 ug/ml for dieldrin.

A plot of the effect of amount of mycelium on pesticide uptake (Fig. 22) demonstrates that the uptake increased linearly with increasing pesticide concentrations between 10 and 100 ug/ml for both aldrin and dieldrin. In either case, the fungus appeared to take up approximately 2000 times the original medium concentration of pesticide; 14 to 17% of the initial pesticide remained unaccounted for.

Other observations of preliminary nature were made on the growth of Z. xylestrix in pesticide media. In one case, when Z. xylestrix was grown on H agar medium, with and without pesticide, it was found that after 3 transfers the fungus grew significantly in liquid H media, with and without pesticides, but not without Tween 80. Finally, it is noteworthy that growth of Z. xylestrix in pesticide-containing media was usually accompanied by morphological changes, such as the conspicuous absence of pellets and lack of normal green pigmentation.

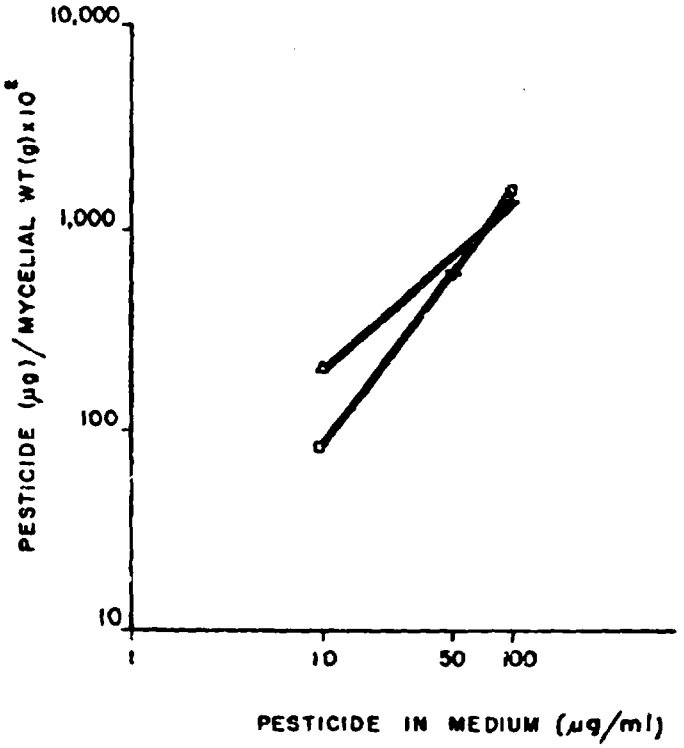


FIG. 22. Uptake of aldrin (Δ) and dieldrin (□) by Z. xylestrix as a function of pesticide concentration in the medium.

DISCUSSION

Most microbiologists have long held the notion that, given enough time, any organic compound can be degraded if exposed to the proper microorganism(s) in a suitable environment. Yet, it is known that bacterial endospores, human hair, tree stumps, rope, and other refractile materials can be found intact after thousands of years. Such material stability over prolonged periods of time may suggest, as Alexander (1965) points out, some unaccessibility of the substrate (or) of some factor essential for growth, which prevents the community of microorganisms from metabolizing the compound because of some physiological inadequacy. Many new organic chemicals used in industry and agriculture appear to possess this property.

Chlorinated hydrocarbon pesticides, among other chemicals, are notorious for persisting in nature. Initially, these compounds appeared to have little effect on non-target organisms, while being very efficient in controlling insects and other invertebrate predators that endangered the health and welfare of man, his crops, and domesticated animals. With the advent of more sophisticated techniques and instrumentation, as well as increased public interest, it has become evident that these chemicals are not inert outside the target organisms. In effect, they interact with microbial populations in areas where they are applied, or to which they are carried by other means, with

results not always beneficial to the ecosystem. As has been mentioned, conversion products resulting from microbial metabolism of pesticides can be more toxic than the parent compounds (Batterton et al ., 1972) and accumulation in primary producers frequently constitutes the first step in the overall process of biological magnification.

Early investigations (Korte, 1962, cited by Brooks, 1959) revealed that, while aldrin and other organochlorine pesticides were attacked by microorganisms in pure culture, dieldrin was not. These findings initiated numerous investigations designed to elucidate the fate of these pesticides, as well as that of their conversion products, in different environments. Successful isolation of pesticide-degrading microorganisms was reported later following microbiological analyses of highly contaminated areas or laboratory systems (Matsumura et al., 1968; Tu et al., 1968).

At the beginning of the work herein reported, it was assumed that adequate information would be available on highly-contaminated aquatic environments elsewhere, as a result of monitoring for pesticides by responsible agencies. Such information, it was anticipated, would facilitate the isolation of microorganisms with enhanced degradation capabilities. Consequently, isolation media and systems were adopted which relied primarily on isolating as many microorganisms as possible and testing these for degradation ability with suitable analytical methods. Differential media

were included only to individually facilitate the analysis of filamentous and non-filamentous microorganisms. In actuality, however, information was sparse and attention had to be restricted to local areas as sources of microorganisms.

Recent data on organochlorine insecticide residues in South Florida (Kolipinski et al., 1971) for example, show very little aldrin in natural waters and man-made canals of this region, a fact now confirmed by our own determinations. Further, unpublished laboratory records made available to the author by the regional office of the Water Resources Division, Geological Survey, U.S. Department of the Interior, failed to show aldrin in any of the samples taken in Dade, Broward, and Palm Beach Counties over a period of several years. Dieldrin was occasionally detected but in concentrations of 0.1 ug/liter or less. A sample taken on 28 February 1969, at the Intracoastal Waterway, Atlantic Boulevard Bridge, Pompano Beach, Florida, however, demonstrated a significant concentration of 0.42 ug/liter.

As far as can be determined, dieldrin usage in Southeastern Florida is limited to rare applications in sugar cane fields around Lake Okeechobee. Aldrin, on the other hand, is used annually, in 10% formulations with fertilizer, on most local vegetable farms. This land drains directly into those canals showing concentrations of dieldrin. It was conceivable, therefore, that dieldrin found in surface waters could have resulted from the microbiological

epoxidation of aldrin (Fig. 1). This, of course, was highly speculative in the absence of actual experimental evidence.

Nevertheless, low pesticide levels in local areas dimmed the prospect of isolating pesticide-adapted microorganisms. A better approach seemed to lie in creating laboratory conditions favoring adaptation of autochthonous microflora by enriching selected water and sediment systems with aldrin and dieldrin.

Another method employed in these studies was based on an observation at the Naval Civil Engineering Laboratory in Port Hueme, California. While working with the shipworm Teredo diegensis, Vind (1971) noticed that pesticide from impregnated matchsticks did not leach out even in hot water, but when submerged in raw seawater, the pesticide rapidly disappeared. It was suggested that pesticide disappearance was due to the metabolic action of cellulolytic organisms growing on the surface of the matchsticks. This system had the added advantage of inducing adaptation under natural conditions. The loss of 65% of the canisters implanted to verify, and perhaps, take advantage of this concept, forced a discontinuation for economic reasons.

The time-honored technique of enrichment was used to select microorgansims adapted or at least showing tolerance to the pesticides. Almost coincident with the start of our work, investigators at the University of Wisconsin had successfully isolated in "H media" a strain of the terrestrial fungus Trichoderma koningi which readily degraded

dieldrin (Bixby, 1971). Our use of this medium in part of the work reported here reflected confidence in the concept and it was hoped that the attack might be carried as far as mineralization.

Originally, the H medium had been devised by Japanese workers to isolate microorganisms capable of utilizing hydrocarbons as carbon sources (Shimahara and Yamashita, 1967). These investigators alternated the use of Tweens, included to disperse insoluble organic substrates, to discourage the organisms from using these long-chain fatty acids instead. Alternation was not followed in these present studies to reduce the variety of isolation media, but Tween 80 was employed as a pesticide dispersant. It was not surprising, therefore, that none of our isolates from this medium grew in the absence of Tween 80 when pesticide was the intended carbon source. This however, did not necessarily indicate that the isolates lacked pesticide-degrading capabilities.

An ecological phenomenon of increasing interest is that of cometabolism or co-oxidation, a term that describes microbial transformation of substrates without utilization of the energy derived (Leadbetter and Foster, 1959; Focht and Alexander, 1970; Horvath and Alexander, 1970). Horvath (1970, 1972a) successfully used an "analog-enriched" medium to isolate microorganisms degrading DDT and herbicides. As he pointed out (1972b), "the existence of co-metabolism represents a severe drawback to the use of growth as a criterion in biodegradability of substrates."

Foster (1972) stated further that one reason for the failure of some microorganisms to grow on certain substrates was the inability to use the oxidation products rather than t · inability to attack the substrate. That some microorganisms, unable to grow in H media, were capable of attacking aldrin and dieldrin causing significant molecular alterations, is clearly seen in our radiolabeled pesticide analyses of grouped cultures. Furthermore, as pointed out above, while none of the H media isolates actually grew with pesticides as sole carbon sources, some produced conversion products.

Although none of the possible products were chemically characterized here, some gave migration values, in identical TLC systems, similar to those reported for aldrin and dieldrin metabolites. For example, spot D (Fig. 5) appeared to be an aldrin-diol while spot D (Fig. 6) was comparable to 6,7-trans-dihydroxydihydro-aldrin (TDDA) (Matsumura, 1968a). Similarly, spot D (Fig. 9) has an R_a comparable to that of spot D (Fig. 6) and to authentic TDDA as reported in the literature. However, these metabolites were found in such small quantities that the possibility of spurious influence by media components or microbial growth products cannot be ruled out.

It is difficult to distinguish between chemical alterations of biological or non-biological nature, particularly when the latter can result from the action of enzymatic systems released by the autolysis of dead organisms (French and Hoppingharner, 1970). Proof of biodegradation by grouped

cultures and other isolates herein, requires actual isolation and chemical characterization of metabolites. Carbon-labeled analyses of grouped and single isolates from media containing aldrin and dieldrin, strongly suggest that microbial action on these pesticides, under optimal culture conditions, may be more extensive than normally assumed. The use of grouped cultures for screening large numbers of isolates and encouraging pesticide degradation, a method not used previously to any large extent, has proven valuable.

The predominance of non-filamentous bacterial isolates from media not made especially favorable for their growth may be significant. Of 14 isolates kept for further study, only three were filamentous fungi and, including those discarded for various reasons, 20 of 24 were non-filamentous. As has been mentioned, quantitative GLC analyses of selected isolates provided little definitive information; yet, higher pesticide recoveries, indicating reduced degradation, were obtained consistently with filamentous organisms. Radioautography confirmed greater activity by non-filamentous organisms. The only exception, was the high activity demonstrated by a grouped culture of filamentous fungi on ^{14}C-labeled dieldrin. The data bear a resemblance to those of other investigators. Malone (1970), for instance, found a higher conversion of DDT to DDD by anchovy-gut bacteria than by fungi isolated from the same specimen. No explanation was given for this phenomenon, but Bollag (1972) has concluded that, although transformation of biopolymers

such as lignin, cellulose, and hemicellulose can be performed best by fungi, pesticide degradation is much more limited in fungi than in bacteria. Present data however, appear insufficient for a statistical opinion on differences in the cyclodiene degradation efficiency of non-filamentous versus filamentous microorganisms. Similarly, the data are inadequate for characterizing the efficiency of the different isolation systems and media.

Observations of samples from System B, Station 04 only hint at information which a more extensive study could provide. Although a reduction of the original pesticide concentration was shown, it could not be attributed to microbial species predominating at any particular time. Mere disappearance of pesticides does not reveal their fate. Microorganisms can adsorb or absorb substrates with or without degradation. Particulate matter or vessel walls also can remove pesticides by simple adsorption.

Work on marine fungi traditionally has been centered on their economic importance. Much has been done on the role of marine fungi in wood and cordage rotting; far less has been done on other possible saprophytic or parasitic roles in the sea. This situation has not improved over the last decade. Bacteria, for example, are known to serve as food for other marine organisms, but this function has only been assumed for fungi. It is known, however, that aging fungi lyse, releasing their sugars, amino acids, and vitamins to the sediment or water.

Initially, the employment of Z. xylostrix in these

studies was not based on high expectations since previous work in this laboratory had shown little versatility in the nutritional requirements of several marine fungal species (Sguros et al., 1972). Now, however, there is no doubt that growth of Z. xylestrix is stimulated by minute concentrations of aldrin or dieldrin. This has been confirmed repeatedly by various methods and under various conditions. At first, it was believed that the organism was utilizing the pesticide in some nutritional manner, but failure of confirmatory experiments, particularly ^{14}C-labeled pesticide analyses cast doubt on the type of interaction involved. The first evidence that substrate assimilation was not solely responsible for the "loss" of pesticides from the medium appeared when cultures were disrupted during extraction and aldrin and dieldrin recoveries increased by 10%. The premise persisted that, since 20-30% of original pesticide (80 ug/ml) disappeared in 15 days incubation, the use of only 10 ug/ml of cyclodiene should result in its complete disappearance. Furthermore, it was believed that increasing glucose concentrations and inoculum size would directly affect pesticide recovery. It was reasoned also that increased rates of pesticide assimilation or disappearance should parallel growth.

When time-course experiments embodying these ideas were performed, using shake cultures containing 10 ug/ml of pesticide, it was found that the rate of uptake increased with time, but recoveries were much higher than anticipated. The presence of glucose appeared inconsequential, as did

inoculum size. In these studies, the term "uptake" (Harold, 1972) has been used to indicate the amount of substrate removed from the medium, without reference to possible transformations occurring during removal. Korte (1967) has reported that a number of cyclodienes were metabolized in inverse proportion to their concentration, but he was investigating assimilation not bioconversion. Extensive TLC analysis of Z. xylestrix failed to demonstrate conversion products that might have accounted for "lost" pesticide.

Subsequent labeled carbon analyses confirmed that the pesticides were not being used as carbon sources. Since the initial substrate concentration in these studies was only 3 ug/ml, disappearance of less than half of the original activity argued against pesticide support of growth. Again, glucose did not appear influential. These results, of course, did not rule out the possibility that some of the activity was lost by CO_2 evolution or incorporation into cell lipid fractions.

While migration rates of possible metabolites, shown by radioautography of Z. xylestrix extracts, correlated well with values in the literature (Matsumura, 1968), chemical characterization is now required before actual metabolism can be confirmed. The photometric scanning which showed less than 15% aldrin - less than 5% dieldrin - recovered as more polar compounds may have been inaccurate since a direct count of radioautographic activity was not made. However, low indications of conversion may be the rule. Matsumura (1968b) found only a 5-10% degradation of 0.5 ug/ml

dieldrin in 30 days, while Tu (1968) reported a 0.01% conversion of 1 ug/ml aldrin to dieldrin. A relatively high conversion of 12% of 20 ug/ml dieldrin in 10 days with E. aerogenes has been obtained by Wedemeyer (1968).

With reference to the observation that with aldrin a higher proportion of metabolites occur than with dieldrin, it cannot be argued that the oxidized state of the pesticide made it more suitable to microbial attack (Brooks, 1969). Most investigators, however, found dieldrin to be more persistent than aldrin and other chlorinated pesticides (Chacho and Lockwood, 1966; Hill and McCarthy, 1967). The unfortunate dieldrin contamination of the original supply of ^{14}C-aldrin, prevented categorization of our cultures with the many life forms which have been reported to epoxidize aldrin to dieldrin. Nevertheless, conversion of aldrin to other products, without the involvement of dieldrin, has been reported (Korte, 1967).

It was not surprising that a more thorough extraction showed that much of the original pesticide has accumulated in the cells. Interestingly, pesticide accumulation in Z. xylostrix increased not only with a greater concentration of pesticide in the medium, but also in direct proportion to total cell mass; in turn, total cell mass increased with a higher pesticide concentration. This may indicate that the correct method of assessing accumulation will require one of the two variables to be fixed, although this probably does not occur in nature.

It is essential to determine how much pesticide is

adsorbed to the cell surface and how much actually passes through the cell membrane. Wheeler (1970) has concluded that radioactive dieldrin was taken up by Chlorella pyrenoidosa in increasing amounts up to a certain time after which the label became more difficult to extract. This suggested that there was a distribution of the label among less easily extracted subcellular organelles and may have explained why 10-15% of original pesticide was still unrecovered even by the most exhaustive extraction procedure.

It seems obvious that this research, particularly that concerning Z. xylestrix, has posed more questions than it has answered. Although difficult techniques and time limitations precluded satisfactory resolution of all problems evoked by the data, it is possible and desirable to elaborate briefly in some of the more pertinent aspects.

Is the enhanced growth observed with Z. xylestrix real or apparent? If it is not an artifact, what causes the stimulation? There is no doubt that the mycelial wt of Z. xylestrix increased in the presence of aldrin or dieldrin. This is not, however, an unprecedented observation. Dalton, Hodkinson, and Smith (1970) conducted experiments with three strains of aquatic Hyphomycetes and found that less than 2 ug/ml of DDT had no effect on the growth as determined by dry mycelial wt. Higher insecticide concentrations, however, enhanced the growth rate. It was suggested that the stimulatory effect could have been due to the use of pesticide as a nutrient, to increased permeability of fungal cell membranes to other nutrients, and/or to increased

metabolic rate because of a cofactor activity of DDT. Foster (1949) has suggested that abnormal environmental conditions provoke "deranged" or pathological behavior in fungi. The introduction of pesticide is easily envisioned as possible abnormal environmental force. Mckenna and Kallio (1964) have suggested that certain hydrocarbons stimulate the endogenous respiration of test organisms without themselves being oxidized. These notions lead to a second question on whether metabolites found in carbon-labeled analyses are the products of microbial metabolism or result from unspecific causes, such as photodecomposition. The following observations provide evidence for a biological process: (1) conversion products were not found in the extensive control standards, (2) when comparing autoradiograms of Z. xylestrix with those of other microbial isolates, the products obtained were not identical in all cases, although some of them did show similar migration values, (3) in general, reproducibility was found in nine determinations performed with dieldrin and six with aldrin. Grouped cultures and H media isolates were examined only once in this connection, but duplicate samples demonstrated high precision.

A puzzling aspect in Z. xylestrix was the lack of correlation among the various extraction procedures used, e.g., whole culture chloroform extraction, with and without insonation, versus separate extraction of cell-free media and washed mycelia. The diversity of solvent systems and pesticide extraction methods reported in the literature,

all claiming recoveries of 85-95% (Mills 1972), made it desirable to adopt a simple standard procedure that could be applied to as many samples as possible. Recoveries obtained from control preparations, with the internal standard, were always consistent with accepted criteria for excellence. Thus, disagreement between extraction methods may have been due to factors in Z. xylestrix cultures which interfered with recovery. Identification of such factors was beyond the scope of this research, but the possible role of Tween 80 in the recovery of accumulated pesticide from fungal cultures should be investigated. It is also recommended that the more thorough Soxhlet extraction method be used in lieu of batch procedures for any future pesticide experimentation involving Z. xylestrix.

Attempts to compare the efficiencies of the different systems and media were also beyond the scope of this study. Similarly, because of the restrictive sample-handling capacity of available equipment, all the isolates could not be screened for possible degradation activity. Therefore, isolates were screened in the minimal medium (H) to select microorganisms with the greatest biodegradation capabilities. These preliminary studies suggest that, where adaptation of microbial populations to pesticides can be achieved by periodic enrichment, controlled laboratory ecosystems represent the most practical approach. When this can be followed by enrichment isolation of pesticide-tolerant microorganisms and screening for metabolic action by radiolabeling or other similarly equivalent sensitive

technique, e.g., GLC with electron capture detector, a fruitful investigation of microbial pesticide degradation will become possible.

Contamination of the environment with persistent pesticides is a reality. The irreparable damage that may result from the process of biological magnification and the decomposition of these chemicals into even more toxic compounds has only begun to be assessed. This research was primarily concerned with establishing methods that would facilitate the study of interactions between aldrin and dieldrin and microorganisms isolated from aquatic environments.

Selection of microbial mutants from highly contaminated laboratory systems, it may be argued, could hardly resemble the process taking place in nature where contamination seldom is expected to reach such levels. However, the fact that these pesticides can be concentrated by microorganisms, as well as by higher forms, to levels many times above that of the environment, may justify the use of exaggerated levels in laboratory systems.

On the other hand, the role of Z. x lestrix in the sea, may be more important than first estimated because of nutritional characteristics. Are these marine fungi only mineralizers or do they also serve as food for higher forms? What are the consequences of autolysis, especially after the fungus has accumulated large quantities of toxic compounds from the environment? For example, Seba (1969) found surface planktonic films in marine environments very

efficient in concentrating persistent chlorinated pesticides, a situation that could parallel the concentrative capabilities of Z. xylestrix. Inasmuch as this role is just beginning to be intensively studied, it seems clear that the ability of Z. xylestrix to accumulate and chemically transform pesticides, may be important in the ecology of the ocean.

SUMMARY

1. Selected locations in surrounding fresh water canals, the brackish Intracoastal Waterway, and the Atlantic littoral zone were monitored for physical and chemical conditions, including the presence of pesticide residues. Levels of aldrin and dieldrin were less than 0.1 ppb in most surface samples except for one fresh water canal which contained 0.6 ppb dieldrin.

2. Microorganisms were isolated using, variously, three isolation systems and selective and/or enriched media. Of 160 isolates, 24 grew in a minimal nutrient medium, containing pesticides, either on primary isolation or after adaptation from grouped cultures.

3. Deliberately mixed axenic isolates also were screened for pesticide interaction in media containing ^{14}C-labeled aldrin or dieldrin by TLC radioautograms of whole culture chloroform extracts. Small amounts of possible metabolites were detected in most of the grouped culture extracts. Spots appeared in definite patterns and their migration values were cataloged.

4. Microorganisms growing in minimal nutrient media were characterized partially using standard identification methods. Most of the non-filamentous organisms were Gram negative, non-fermentative rods. Two of the filamentous organisms were identified tentatively as Trichoderma sp. and a third as a Monocillium sp.

5. Microbial action on labeled pesticides was determined

by TLC procedures on whole culture extracts. Selected isolates also were tested by quantitative GLC procedures. Neither TLC nor GLC analyses provided complete information on the degradation potentials of the cultures.

6. Radioautographic analyses of chloroform extracts of whole cultures containing ^{14}C-labeled aldrin or dieldrin revealed decomposition products in the case of seven organisms. Migration values for some of these products were identical to those in grouped culture extracts. Identification of the products was not possible by comparison with data in the literature.

7. One isolation system (Station 04, System B) was held for several months in the laboratory. The level of pesticide initially added, as monitored by GLC and spectrophotometry, decreased significantly, but attempts to correlate this with observed microbial successions were inconclusive.

8. In general, isolation from pesticide-enriched, laboratory-simulated ecosystems followed by labeled pesticide screening, represented the best means of acquiring microorganisms with degradative potential.

9. Cellulolytic Z. xylestrix, from the stock collection, grew well in basal media at concentrations of aldrin or dieldrin of 10-250 ug/ml. Inhibition was complete above 500 ug/ml. Maximal growth occurred in 10 days and was unaffected by previous exposure to the pesticides.

10. Higher yields of Z. xylestrix mycelial wt and cell N were obtained in pesticide-containing media, with and

without glucose, than with pesticide-free cultures. These results indicated that ug/ml concentrations of pesticide had stimulatory effects on Z. xylestrix growth.

11. Quantitative analysis (GLC) of whole mycelial extracts of Z. xylestrix demonstrated a significant disappearance of pesticide, but qualitative analysis of the same extracts by TLC, using various supports and mobile phases, failed to suggest decomposition products.

12. Radioautographic analysis (TLC) of pesticide-containing Z. xylestrix demonstrated small amounts of possible decomposition products.

13. A better procedure, using separate extractions for washed mycelia and cell-free media, demonstrated that Z. xylestrix achieved 2000-fold accumulations of pesticide. The mycelial uptake of both aldrin and dieldrin increased linearly with their increasing concentrations in the medium.

14. Usually, 5-15% of the pesticide remained unaccounted for in Z. xylestrix culture extracts using labeled and unlabeled pesticides, at high or low concentrations, and with different extraction procedures.

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APPENDIX I

ANALYTICAL PROCEDURE FOR ANALYSIS OF WATER FOR PESTICIDES

PRIMATE RESEARCH LABORATORY [1]

It is assumed that final TLC and microcoulometric confirmation may be applied to supplement the information obtained by electron capture detection. For this reason a larger sample is used than would be necessary for E.C. Dilution of an aliquot of the final extract for E.C. GLC requires far less time than the extraction of another sample.

1. Transfer 3 L. of sample (or a lesser volume, if indicated) to 4 L. sep. funnel and add 150 ml of 15% ethyl ether/hexane.

   NOTE: If, on the basis of prior analysis of a given waterway, the residue levels may be expected to run high, a sample of one liter may be indicated. In this event, the size of the sep funnel should be 2-liters and the extraction solvent volumes given as 150 ml should be reduced to 60 ml. In further connection with this reduction, the extract should be filtered through the Na_2SO_4 directly into the Kuderna-Danish flask instead of into a beaker (steps 4-9).

2. Stopper funnel and shake vigorously 2 minutes. Allow layers to separate and draw off aqueous layer into a second 4 L. sep. funnel.

3. Add another 150 ml of 15% EE/hexane to the aqueous phase in sep. funnel #2, stopper and shake vigorously another 2 minutes.

4. Prepare a 2-inch column of anhydrous, granular Na_2SO_4 in a 150 x 24 mm filter tube with a small wad of pre-extracted glass wool at the bottom. Position this over a 600-ml beaker.

5. Filter the EE/hexane extract in sep. funnel #1 through the Na_2SO_4 column into the beaker.

[1] Adopted from: Manual of Analytical Methods, Pesticide Community Studies Laboratories, Primate Research Laboratory, Environmental Protection Agency, Perrine, Florida. 1971. Verbatim copy of text does not conform in style to rest of report.

6. Draw off the aqueous layer in sep. funnel #2 into empty sep. funnel #1.

7. Add 150 ml of straight hexane to the aqueous solution in sep. funnel #1, stopper and shake again for 2 minutes. Draw off and discard the aqueous layer.

8. Filter the solvent extracts in both sep. funnels through the Na_2SO_4 into the beaker, rinsing down filter tube with three 10 ml portions of hexane.

9. Place the beaker containing the combined extracts in a water bath adjusted to 70°C. and evaporate under a nitrogen stream to ca 250 ml.

10. Prepare a Kuderna-Danish evaporation assembly consisting of 500 ml K-D flask fitted with a 10 ml grad concentrator tube containing one 3 mm glass bead.

11. Add ca 5 grams anhydrous Na_2SO_4 to the cooled solvent extract in the beaker and distribute with a glass stirring rod. Transfer the extract through a glass funnel into the K-D flask rinsing beaker with three 10-ml portions of hexane applied with a pipet so that the rinse stream can be easily directed.

12. Attach a 3-ball Snyder column to the K-D flask, place assembly in a boiling water bath and concentrate extract to 3-4 ml.

13. Remove K-D assembly from bath, cool and rinse ⧧ joint between tube and flask with a small volume of hexane and adjust volume in tube to exactly 10 ml.

14. Stopper concentrator tube and mix on Vortex mixer for 1 minute.

GAS CHROMATOGRAPHY

The initial GLC determination will be conducted using E.C. detection with the working columns and operating parameters in Section 4, B of this manual. An exploratory 5 ul injection of the 10 ml concentrated extract should serve to inform the chromatographer of the degree of dilution (if required) that may be necessary to bring the peaks into a quantifiable range consistent with the linear range of the detector. When this has been determined, a 1 ml aliquot should be withdrawn from the 10 ml of extract concentrated and diluted with hexane in a volumetric flask or concentrator tube to an appropriate volume.

If the initial chromatogram indicates the presence of a sufficient amount of interfering materials, it may prove necessary to conduct a Florisil cleanup on the extract. Based on the general experience of water chemists, this is rarely necessary on most surface water samples. If it should prove necessary, the cleanup should be carried out as prescribed in Section 5, A (1), page 7 of this manual. After the cleanup, another exploratory injection is made followed by peak identifications and quantitation.

The chromatographer must exercise his judgement in determining from the information provided by E.C. detection whether further confirmation will be required to establish the identities of the compounds as indicated by E.C. Microcoulometry and especially thin layer chromatography should provide the tools for most positive identifications. The TLC technique is fully described in this manual in Section 12, B. If the relative retention values of suspect peaks by E.C. match the values listed in the RR tables of Section 4, B for any of the organophosphate compounds, final identification may be established by TLC or by flame photometric detection, if available.